University of Alberta

Regulation of Fatty Acid Metabolism:
Role of Leptin, Exercise and Insulin Resistance

by

Laura Lee Atkinson ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirements for the degree of Doctor of Philosophy

in

Medical Sciences - Pediatrics

Edmonton, Alberta

Spring 2003

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisisitons et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 0-612-82079-3
Our file Notre référence
ISBN: 0-612-82079-3

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou aturement reproduits sans son autorisation.

Canada

University of Alberta

Library Release Form

Name of Author: Laura Lee Atkinson

Title of Thesis: Regulation of Fatty Acid Metabolism: Role of Leptin, Exercise and Insulin Resistance

Degree: Doctor of Philosophy

Year this Degree Granted: 2003

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

Dec 23, 2002

L Atkinson
Laura Atkinson
306-9925 91 Avenue
Edmonton, Alberta
T6E 2T7

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Regulation of Fatty Acid Metabolism: Role of Leptin, Exercise and Insulin Resistance submitted by Laura Lee Atkinson in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences – Pediatrics.

Dr. Gary D. Lopaschuk

DN Brindley

Dr. David N. Brindley

Dr. Alexander S. Clanachan

Jason Dyck

Dr. Jason RB. Dyck

Dr. James C. Russell

Dr. Arend Bonen

Dec 20, 2002

Dedication

This thesis is dedicated to my mother, Doreen Marie Atkinson and in loving memory of my father, Gerald Ross Atkinson, who passed away on September 22, 2002 after a long, courageous battle with cancer.

ABSTRACT

There is a strong link between insulin resistance and cardiovascular disease. However, it is not clear whether the heart becomes resistant to the effects of insulin or whether alterations in fatty acid metabolism contribute to insulin resistance. Since AMP-activated protein kinase (AMPK) plays a key role in the regulation of fatty acid and glucose metabolism, we investigated whether a reduction in AMPK activity is associated with altered fatty acid and glucose metabolism in insulin resistance. Leptin regulates fatty acid metabolism in liver, skeletal muscle and pancreas by partitioning fatty acids into oxidation rather than triacylglycerol storage. We demonstrate for the first time that leptin significantly activates fatty acid oxidation and decreases TG content in the heart, independent of changes in AMPK activity, acetyl-CoA carboxylase (ACC) activity or malonyl-CoA levels. Isolated working hearts from leptin receptor-deficient JCR:LA-cp rats were used to demonstrate that insulin resistant hearts have elevated cardiac triacylglycerol (TG) content associated with a reduction in glucose metabolism. However, we observed no difference in fatty acid oxidation rates or change in GPAT activity, the first step of TG synthesis. This suggests that increased fatty acid supply or decreased TG lipolysis rather than reduced fatty acid oxidation contribute to cardiac TG accumulation in the insulin resistant rat. We extended our studies to investigate potential alterations in ACC and AMPK in the liver and skeletal muscle of the JCR:LA-cp rat during the development of insulin resistance. We demonstrate that, like heart, alterations in ACC and AMPK activity in muscle do not contribute to the development of insulin resistance. However, increased hepatic ACC in the JCR:LA-cp rat may contribute to the development of lipid abnormalities, since the lipid-lowering agent, MEDICA 16 significantly reduced hepatic ACC activity. We further demonstrate that exercise improves whole body insulin sensitivity and activates cardiac AMPK. The exercise-induced activation of cardiac AMPK was associated with a reduction in TG content and an increase in glycolysis. In summary, increased fatty acid supply contributes to TG accumulation and the development of insulin resistance in the JCR:LA-cp rat heart and skeletal muscle. The activation of AMPK activity is associated with a correction of the altered fatty acid and glucose metabolism in the insulin resistant JCR:LA-cp rat.

TABLE OF CONTENTS

Page

1. Introduction 1

Obesity, Insulin Resistance and Diabetes Mellitus 2

Insulin Resistant Syndrome 3

Overview of Fatty Acid Metabolism 5

Overview of Glucose Metabolism 8

Role of Fatty Acids in the Development of Insulin Resistance 11

Role of Substrate Competition in the Development of Insulin Resistance 12

Role of Triacylglycerol Accumulation in the Development of Insulin Resistance 13

Coordinated Regulation of Fatty Acid Metabolism 16

Coordinated Regulation of Fatty Acid and Glucose Metabolism ... 16

Hormonal Regulation of Fatty Acid Metabolism 21

Leptin 21

Adiponectin 23

Cardiac Metabolism and Insulin Resistance 24

Experimental Models of Insulin Resistance 25

Modification of the Insulin Resistant Syndrome 27

Exercise Training and Insulin Sensitivity 28

Models of Exercise Training 29

Hypothesis and Objectives 30

References 32

2. Materials and Methods 55

- Materials 56
- Methods 57
  - Sprague Dawley Rats 57
  - JCR:LA-cp Rats 57
  - JCR:LA-cp Rats (Exercise Study) 58
  - MEDICA 16 Treatment 58
  - Blood Collection 59
  - Plasma Measurements 59
  - Meal Tolerance Test 59
  - Exercise Protocol 60
  - Wheel Revolutions 61
  - Heart Perfusions 61
  - Perfusion Protocols 62
  - Tissue Analysis 63
  - Tissue Collection 64
  - Tissue Triacylglycerol Determination 64
  - [^{3}H]Palmitate Incorporation into Triacylglycerol 65
  - CoA Ester Levels 66
  - Polyethylene Glycol (PEG) Precipitation 66
  - Whole Cell Homogenate Preparation 67
  - ACC Activity 68
  - AMPK Activity 68
  - GPAT Activity 69
  - Preparation of Mitochondria 70
  - CPT-I Activity 70
  - Immunoblotting 71
  - Densitometric Analysis 72
  - Statistical Analysis 73
- References 74

3. Leptin Activates Cardiac Fatty Acid Oxidation Independent of Changes in the AMP-Activated Protein Kinase-Acetyl-CoA-Malonyl-CoA Axis 77
  Abstract 78
  Introduction 80
  Methods 82
    Leptin Dose 82
    Heart Perfusions 82
    [^{3}H] Palmitate Incorporation into Triacylglycerol 83
    Tissue Extractions 84
    Immunoblotting 84
    Statistical Analysis 84
  Results 85
  Discussion 101
  Summary 106
  References 107

4. Potential Mechanisms and Consequences of Cardiac Triacylglycerol Accumulation in Insulin Resistant Rats 112
  Abstract 113
  Introduction 115
  Methods 117
    Animals 117
    Plasma Measurements 117
    Heart Perfusions 118
    Tissue Collection 119
    Tissue Extractions 119
    Immunoblotting 119
    Statistical Analysis 119

Results 121
Discussion 135
Summary 140
References 141

5. MEDICA 16 Inhibits Hepatic Acetyl-CoA Carboxylase and Reduces Plasma Triacylglycerol Levels in Insulin Resistant Rats 146
  Abstract 147
  Introduction 149
  Methods 152
    Animals 152
    MEDICA 16 Treatment 152
    Tissue and Blood Isolation 152
    Plasma Measurements 153
    Tissue Extractions 153
    Immunoblotting 153
    Statistical Analysis 153
  Results 154
  Discussion 172
  Summary 179
  References 180

6. Chronic Exercise Improves Whole Body Insulin Sensitivity in Insulin Resistant Rats: Role of AMP-Activated Protein Kinase in the Regulation of Cardiac Triacylglycerol Content and Glycolysis 187
  Abstract 188
  Introduction 191

Methods 194
    Animals 194
    Tissue Collection 195
    Plasma Measurements 196
    Heart Perfusions 196
    Tissue Extractions 197
    Statistical Analysis 197
Results 198
Discussion 221
Summary 225
References 226

7. Discussion and Conclusions 233
    Leptin Regulation of Cardiac Fatty Acid Metabolism 234
    Potential Mechanisms of Cardiac Insulin Resistance 236
    Role of AMPK in Insulin Resistance and Potential Target for Treatment 239
    Future Directions 241
    Limitations 243
    References 246

LIST OF TABLES

Table 3-1. The effect of leptin on mechanical function of isolated working rat hearts 91

Table 3-2. The effect of leptin on cardiac ACC activity and malonyl-CoA levels 92

Table 3-3. The effect of leptin + insulin on mechanical function of isolated working rat hearts 93

Table 3-4. The effect of leptin + insulin on oxidative rates, TG content and AMPK-ACC-malonyl-CoA........ 94

Table 4-1. Characteristics of 12 week lean and insulin resistant rats 126

Table 4-2. Mechanical function of isolated working rat hearts from 12 week old lean and insulin resistant rats........ 127

Table 4-3. Plasma lipid levels and cardiac FABP protein expression in 12 week old lean and insulin resistant rats........ 128

Table 5-1. Body weight and plasma measurements of 4 and 12 week lean and JCR:LA-cp rats 161

Table 5-2. Body weight and plasma measurements of 12 week JCR: LA-cp and MEDICA 16-treated JCR:LA-cp rats 162

Table 6-1. Fasting plasma fatty acid and TG levels 204

Table 6-2. Mechanical function of isolated working JCR:LA-cp rat hearts 205

Table 6-3. Heart weight:body weight ratios 206

Table 6-4. Body weight and food intake at 12.5 weeks of age following 8 week experimental protocol 207

Table 6-5. Fasting plasma measurements at 12.5 weeks of age following 8 week experimental protocol 208

Table 6-6. Parameters of fatty acid and glucose metabolism at 12.5 weeks of age following 8 week experimental protocol 209

Table 6-7. Mechanical function and heart weight:body weight ratio of isolated working JCR:LA-cp rat hearts at 12.5 weeks of age following 8 week experimental protocol 210

LIST OF FIGURES

Figure 1-1. Regulation of mitochondrial fatty acid uptake 7

Figure 1-2. The role of phosphofructokinase-1 in the regulation of glycolysis 10

Figure 1-3. Role of AMPK in the regulation of fatty acid and glucose metabolism 20

Figure 3-1. The effect of 60 ng/ml leptin on rates of palmitate oxidation and glucose oxidation 95

Figure 3-2. The effect of 60 ng/ml leptin on total cardiac triacylglycerol content and palmitate incorporation into triacylglycerol 96

Figure 3-3. The effect of 60 ng/ml leptin on total exogenous and endogenous fatty acid oxidation and total TCA acetyl-CoA production 97

Figure 3-4. The effect of 60 ng/ml leptin on oxygen consumption and the efficiency of cardiac energy production 98

Figure 3-5. The effect of 60 ng/ml leptin on cardiac AMPK and Thr-172 phosphorylation of AMPK in hearts exposed to leptin for 60 minutes 99

Figure 3-6. The effect of 60 ng/ml leptin on cardiac AMPK and Thr-172 phosphorylation of AMPK in hearts exposed to leptin for 5 and 10 minutes 100

Figure 4-1. Glycolytic rates in isolated working hearts from lean and insulin resistant rats 129

Figure 4-2. Palmitate oxidation rates in isolated working hearts from lean and insulin resistant rats under fasting and fed conditions 130

Figure 4-3. Cardiac triacylglycerol content in lean and insulin resistant rats 131

Figure 4-4. Cardiac AMPK activity in lean and insulin resistant rats ... 132

Figure 4-5. Cardiac ACC activity and malonyl-CoA levels in lean and insulin resistant rats 133

Figure 4-6. Cardiac CPT-1 activity and GPAT activity in lean and insulin resistant rats 134

Figure 5-1. Triacylglycerol content of liver and skeletal muscle of JCR:LA-cp rats and lean age-matched controls 163

Figure 5-2. Hepatic ACC activity and protein expression in JCR:LA-cp rats and lean age-matched controls 165

Figure 5-3. Hepatic AMPK activity measured in JCR:LA-cp rats and lean age-matched controls 166

Figure 5-4. Skeletal muscle ACC activity and protein expression in JCR:LA-cp rats and lean age-matched controls 168

Figure 5-5. Skeletal muscle AMPK activity measured in JCR:LA-cp rats and lean age-matched controls 169

Figure 5-6. Effect of MEDICA 16 treatment on liver ACC and AMPK activity 170

Figure 5-7. Effect of MEDICA 16 treatment on skeletal muscle ACC and AMPK activity 171

Figure 6-1. 4 and 8 week experimental protocols 211

Figure 6-2. Wheel revolutions ran by the exercise-trained JCR:LA-cp rats 212

Figure 6-3. Body weight and food intake in exercise-trained, weight-paired and sedentary control groups 213

Figure 6-4. Fasting plasma insulin in the exercise-trained, weight-paired and sedentary control groups 214

Figure 6-5. Insulin sensitivity in the exercise-trained, weight-paired and sedentary control groups 215

Figure 6-6. Cardiac AMPK activity, glycolytic rates and TG content in exercise-trained, weight-paired and sedentary control groups 217

Figure 6-7. Cardiac palmitate oxidation, ACC activity and malonyl-CoA levels in exercise-trained, weight-paired and sedentary control groups 219

Figure 6-8. Fasting plasma insulin levels and insulin sensitivity in exercise-trained, weight-paired and sedentary control groups at 12.5 weeks of age following 8 week experimental protocol............ 220

LIST OF SYMBOLS AND ABBREVIATIONS

α: alpha
β: beta
γ: gamma
^{O}C: degrees celsius
%: percent
ACBP: acyl-CoA binding protein
ACC: acetyl coenzyme A carboxylase
acetyl-CoA: acetyl coenzyme A
ACRP30: adipocyte complement-related protein
AICAR: aminoimidazole-4-carboxamide-1-beta-D-riboside
AMARA: synthetic peptide (AMARAASAAALARRR)
AMP: adenosine monophosphate
AMPK: 5'adenosine monophosphate-activated protein kinase
AMPKK: 5'adenosine monophosphate-activated protein kinase kinase
ATP: adenosine triphosphate
BSA: bovine serum albumin
C: carbon
^{14}C: radiolabelled carbon
$CaCl_2$: calcium chloride
cAMP: cyclic adenine monophosphate
CAT-1: carnitine acyltranslocase
CO_2: carbon dioxide
CoA: coenzyme A
CPT-I: carnitine palmitoyltransferase-I
CPT-II: carnitine palmitoyltransferase-II
Cr: creatine

db: gene encoding mutation in leptin receptor
ddH_2O: double distilled water
DG: diacylglycerol
DGAT: diacylglycerol acyltransferase
DTT: dithiothreitol
EDTA: ethylenediamine-tetraacetic acid
EGTA: ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
fa: gene encoding mutation in leptin receptor
FA: fatty acid
FABP: fatty acid binding protein
$FADH_2$: flavin adenine dinucleotide
g: gram
GLUT-2: glucose transporter-2
GLUT-4: glucose transporter-4
GPAT: glycerol-3-phosphate acyltransferase
^3H: tritium
H_2O: water
H_3PO_4: phosphoric acid
HCl: hydrochloric acid
HEPES: 4-(2-hydroxyethyl) piperazine-1-ethane sulphonic acid
HPLC: high performance liquid chromatography
hr: hour
HSL: hormone sensitive lipase
IDDM: insulin deficient diabetes mellitus
JAK: janus kinase
KCl: potassium chloride
KCN: potassium cyanide
K_2HPO_4: potassium phosphate dibasic
kg: kilogram

l: liter

LPA: lysophosphatidic acid

LPL: lipoprotein lipase

malonyl-CoA: malonyl coenzyme A

MAPK: mitogen activated protein kinase

MEDICA 16: beta, beta'-tetramethylhexadecanedioic acid

mg: milligram

$MgCl_2$: magnesium chloride

$MgSO_4$: magnesium sulfate

ml: milliliter

min: minute

mM: millimolar

mmHg: millimeters of mercury

N_2: nitrogen

Na^+: sodium

NaCl: sodium chloride

NADH: nicotinamide adenine dinucleotide

NaF: sodium fluoride

ng: nanogram

nmol: nanomole

$NaHCO_3$: sodium bicarbonate

NEM: N-ethylmaleimide

NIDDM: non-insulin-dependent diabetes mellitus

nmol: nanomole

O_2: oxygen

ob: gene encoding leptin protein

^{32}P: radiolabelled phosphorus

PA: phosphatidic acid

PAP: phosphatidic acid phosphohydrolase

PCr: phosphocreatine
PEG: polyethylene glycol
PDH: pyruvate dehydrogenase
PFK-1: phosphofructokinase-1
PFK-2: phosphofructokinase-2
PKA: protein kinase A
pmol: picomole
PPARα: peroxisome proliferator activated receptor alpha
PSP: peak systolic pressure
PVDF: polyvinylidene difluoride
SAMS: synthetic peptide (HMRSAMSGLHLVKRR)
SDS-PAGE: sodium dodecylsulphate polyacrylamide gel electrophoresis
SE: standard error
STAT: signal transducers and activators of transcription
TCA: tricarboxylic acid
TG: triacylglycerol
Thr-172: site of phosphorylation, residue threonine 172
TNFα: tumor necrosis factor alpha
μL: microliter
μmol: micromole
μU: microunit
VLDL: very-low-density lipoprotein
wt: weight

CHAPTER 1. INTRODUCTION

CHAPTER 1. INTRODUCTION

Obesity, Insulin Resistance, and Diabetes Mellitus

The incidence of obesity is increasing at such an alarming rate that obesity is now considered a worldwide epidemic (1). Recent estimates suggest that 50% of Canadians are overweight (61% of Americans) and 15% are obese (26% of Americans) (2,3). Obesity and overweight are commonly identified using body mass index (BMI) which is calculated as height in meters divided by weight in kilograms squared. Overweight is classified as a BMI between 25 and 30 while obesity is classified as a BMI above 30. Obesity and overweight are considered important risk factors for the development of insulin resistance and type 2 diabetes (4). Thus, the rapid increases in both adult and youth obesity have resulted in simultaneous increases in the incidences of insulin resistance and type 2 diabetes (5-7).

There are two types of diabetes: type 1 diabetes characterized by insulin deficiency (IDDM; insulin deficient diabetes mellitus) and type 2 diabetes characterized by resistance to the effects of insulin (NIDDM; non-insulin-dependent diabetes mellitus). Type 1 diabetes can be treated by insulin therapy and accounts for 5-10% of diabetes cases. However, type 2 diabetes which accounts for 90-95% of diabetes cases is generally treated by weight loss, exercise and agents that lower plasma lipid levels and/or increase insulin sensitivity.

The insulin resistant syndrome is a pre-type 2 diabetic state characterized by obesity, hyperinsulinemia, hyperlipidemia and impaired insulin-stimulated glucose metabolism [reviewed in 8-10]. The development of insulin resistance is a prerequisite for the progression to type 2 diabetes (4). Type 2 diabetes occurs when plasma glucose levels become hyperglycemic due to failure of the pancreas to maintain hyperinsulinemia (11)

Significantly, cardiovascular disease is strongly associated with obesity, insulin resistance and type 2 diabetes. Obesity is an important risk factor for cardiovascular disease (12-15), increasing the risk of myocardial infarction by up to 55% (16). Furthermore, the insulin resistant syndrome results in a high risk for the development of atherosclerosis, angina, acute myocardial infarction and congestive heart failure (17-20).

An understanding of the mechanisms involved in the development of insulin resistance is crucial for the development of clinical agents. The availability of effective agents to treat insulin resistance should not only reduce the risk of cardiovascular disease, but will also prevent the progression to type 2 diabetes and the complications that accompany this disease.

Insulin Resistant Syndrome

The insulin resistant syndrome is characterized by obesity, hyperinsulinemia and hyperlipidemia [reviewed in 8-10]. Whole body insulin resistance involves several target organs including skeletal muscle, liver, adipose tissue and pancreas.

The key features of insulin resistance are reduced insulin-stimulated glucose uptake, increased hepatic glucose production, increased synthesis and secretion of very low density lipoproteins (VLDL), elevated plasma fatty acids and hypersecretion of insulin. Hyperinsulinemia results as a compensatory response due to the resistance of insulin-mediated glucose uptake in target insulin sensitive tissues (21).

The presence of cardiac insulin resistance in hypertensive (22) and aged rats (23), as well as in patients with angina (24), heart failure and coronary heart disease (25) has demonstrated a link between insulin resistance and cardiovascular disease. However, relatively few studies have examined whether the development of whole body insulin resistance is also accompanied by reduced insulin-stimulated glucose metabolism at the level of the heart (25,26).

Altered fatty acid and glucose metabolism plays a key role in the development of insulin resistance. Thus, an overview of fatty acid and glucose metabolism will be given prior to discussion of the potential mechanisms involved in the development of insulin resistance. Key regulatory pathways in fatty acid and glucose metabolism will be discussed with specific focus on AMP-activated protein kinase and its potential as a novel target in the treatment of the insulin resistance.

Overview of Fatty Acid Metabolism

Fatty acids are an important source of energy for most cells. Fatty acids are transported in the plasma bound to albumin or in lipoproteins (VLDL and chylomicrons). Lipoprotein lipase (LPL), situated on the luminal wall of capillaries, catalyzes the hydrolysis of triacylglycerol (TG) in VLDL and chylomicrons, releasing fatty acids which are taken up primarily by heart, skeletal muscle and adipose tissue. Fatty acids are taken up by diffusion or via protein-mediated transport. Fatty acid-binding protein (FABP(pm)), fatty acid transport protein (FATP) and fatty acid translocase (FAT/CD36), are membrane-associated proteins involved in the transport of fatty acids [reviewed in 27]. Fatty acids are bound to cytosolic fatty acid binding proteins (FABP) and activated fatty acids can bind to acyl-CoA binding proteins (ACBP). In order for fatty acids to be metabolized, fatty acids must be activated by acyl-CoA synthetase which converts the carboxylic acid moiety to a CoA thioester. Fatty acids can then be oxidized in mitochondria or be used in the synthesis of complex lipids such as TG.

The uptake of fatty acids into mitochondria is controlled by the carnitine shuttle and is depicted in Fig. 1-1. The carnitine shuttle consists of carnitine acyltranslocase (CAT-1), carnitine palmitoyltransferase I (CPT-I) and carnitine palmitoyltransferase II (CPT-II). CPT-I is located in the outer mitochondrial membrane and converts fatty acyl-CoA to fatty acylcarnitine. Fatty acylcarnitine is translocated across the inner mitochondrial membrane by (CAT-1), located on the outer surface of the inner mitochondrial membrane. Fatty acylcarnitine is then converted back to fatty acyl-CoA in the matrix by carnitine palmitoyltransferase II (CPT-II). Two isoforms of CPT-I exist (28): a liver isoform (L-CPT-I) and a muscle isoform (M-CPT-I). Both isoforms are subject to regulation by malonyl-CoA; however, the M-CPT-I is 100 times more sensitive to malonyl-CoA (29).

Once inside mitochondria, fatty acids enter the fatty acid oxidation spiral. Each turn of the beta oxidation spiral removes one acetyl-CoA, eventually degrading the fatty acid to acetyl-CoA. Acetyl-CoA enters the tricarboxylic acid cycle (TCA cycle) also known as Krebs' cycle or citric acid cycle. The TCA cycle is regulated by the energy status of the cell and consists of eight sequential reactions that convert acetyl-CoA to carbon dioxide and the reducing equivalents, NADH and $FADH_2$. NADH and $FADH_2$ subsequently enter the electron transport chain which uses molecular oxygen to produce ATP.

Although adipose tissue is considered the major storage site of TG, most cells contain small amounts of TG that is in constant turnover and provides energy when exogenous supplies are decreased. Glycerol-3-phosphate acyltransferase (GPAT) is the first committed step in *de novo* TG synthesis. There are 2 isoforms of GPAT: mitochondrial GPAT and microsomal GPAT. GPAT transfers acyl-CoA to glycerol-3-phosphate resulting in lysophosphatidic acid (LPA). LPA is converted to phosphatidic acid (PA) which can then be converted to diacylglycerol (DG) through the action of phosphatidic acid phosphohydrolase (PAP). DG can also be converted to TG through diacylglycerol acyltransferase (DGAT). The hydrolysis or breakdown of TG is catalyzed by hormone sensitive lipase (HSL).

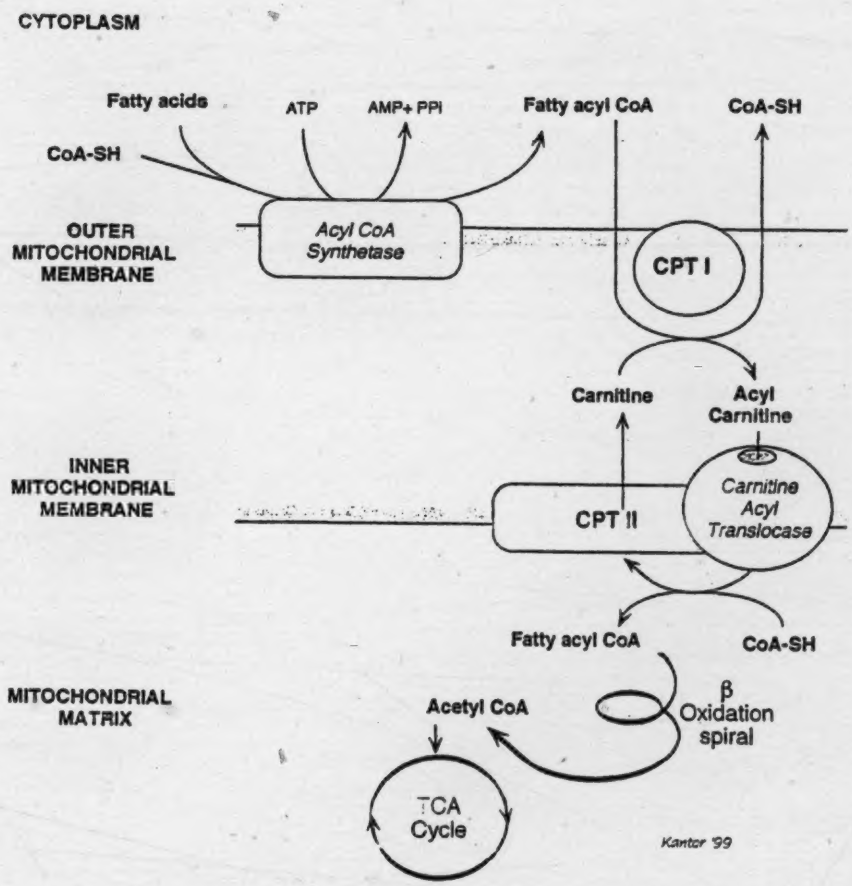

CYTOPLASM
Fatty acids
CoA-SH
ATP
AMP+ PPi
Fatty acyl CoA
CoA-SH
OUTER MITOCHONDRIAL MEMBRANE
Acyl CoA Synthetase
CPT I
Carnitine
Acyl Carnitine
INNER MITOCHONDRIAL MEMBRANE
CPT II
Carnitine Acyl Translocase
Fatty acyl CoA
CoA-SH
MITOCHONDRIAL MATRIX
Acetyl CoA
β Oxidation spiral
TCA Cycle
Kantor '99

Figure 1-1. Regulation of mitochondrial fatty acid uptake. Taken from Kantor PF, Lopaschuk GD, Opie LH: Myocardial Energy Metabolism. In *Heart Physiology and Pathophysiology*, 4th Ed. Academic Press 2001, p. 543-569.

Overview of Glucose Metabolism

Glucose enters the cell via carrier-mediated membrane transporters that move glucose down a concentration gradient into the cell. Glucose is rapidly converted to glucose-6-phosphate catalyzed by glucokinase in liver and hexokinase in muscle. In muscle, insulin stimulates glucose uptake through translocation of the glucose transporter (GLUT-4) to the plasma membrane. The intrinsic activity of GLUT-4 at the plasma membrane may also be increased through phosphorylation by p38 mitogen-activated protein kinase (MAPK) (30). In liver, insulin increases the synthesis of glucokinase with no effect on glucose transporters (GLUT-2).

Glucose-6-phosphate then enters glycolysis or 'the Embden-Myerhof' pathway. Glycolysis is the central pathway of glucose metabolism and occurs in all cells. The primary site of regulation in the glycolytic pathway is phosphofructokinase-1 (PFK-1) depicted in Fig. 1-2. PFK-1 is activated by AMP and fructose-2,6-bisphosphate. Fructose-2,6-bisphosphate is formed by the action of phosphofructokinase-2 (PFK-2). Both protein kinase A (PKA) and AMPK phosphorylate PFK-2 resulting in a stimulation of glycolysis. The end product of the ten reactions catalyzed in the glycolytic pathway is pyruvate. Pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase (PDH). PDH activity is highly regulated by the energy status of the cell. Accordingly, PDH is inhibited by acetyl-CoA, NADH and ATP, products of fatty acid oxidation. PDH is also inhibited by phosphorylation by its kinase (PDH kinase). PDH kinase is activated by acetyl-CoA, NADH and ATP, ensuring that PDH activity is minimal in the presence of high energy content. Acetyl-CoA produced by PDH then enters the mitochondrial TCA cycle to produce NADH and $FADH_2$, which ultimately lead to the production of ATP.

Glucose can also be converted to its storage form, glycogen, following uptake into the cell. Glycogen is present in almost all cells, but is highly abundant in muscle and liver. Glycogen, like TG, undergoes constant turnover, and provides an energy source when exogenous sources are low. Glycogen synthesis and degradation are regulated by glycogen synthase and glycogen phosphorylase, respectively.

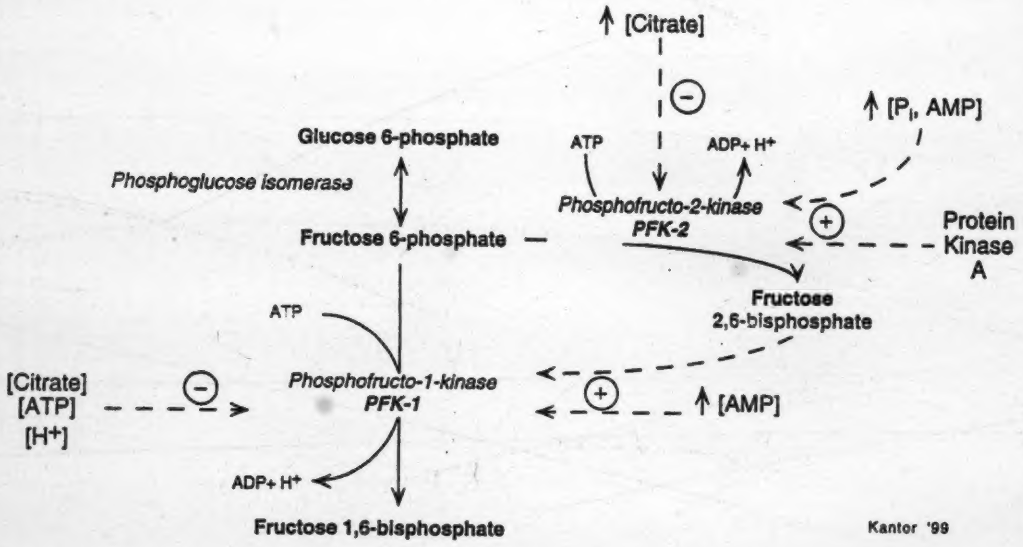

↑ [Citrate]
−
↑ [P_i, AMP]
Glucose 6-phosphate
ATP
ADP+ H+
Phosphoglucose isomerase
Phosphofructo-2-kinase
PFK-2
+
Protein Kinase A
Fructose 6-phosphate
Fructose 2,6-bisphosphate
ATP
[Citrate]
[ATP]
[H+]
−
Phosphofructo-1-kinase
PFK-1
+
↑ [AMP]
ADP+ H+
Fructose 1,6-bisphosphate
Kantor '99

Figure 1-2. The role of phosphofructokinase-1 in the regulation of glycolysis. Taken from Kantor PF, Lopaschuk GD, Opie LH: Myocardial Energy Metabolism. In *Heart Physiology and Pathophysiology*, 4th Ed. Academic Press 2001, p. 543-569.

Role of Fatty Acids in the Development of Insulin Resistance

The strong association of obesity with insulin resistance (31) suggests that fatty acids could play a causative role in the development of insulin resistance [reviewed in 32]. Obesity results in an increased availability of plasma fatty acids (33,34); the result of increased adipose tissue mass and/or insensitivity to the inhibitory action of insulin on lipolysis (35,36). Elevated plasma fatty acids result in increased synthesis and secretion of VLDL, and a reduced ability of insulin to suppress hepatic glucose production [reviewed in 32,37]. Fatty acids have been demonstrated to interfere with glucose utilization in skeletal muscle and heart through the glucose-fatty acid cycle proposed by Randle (38-40). The hypersecretion of insulin by the pancreas is induced acutely by fatty acids [reviewed in 41]. Chronic exposure of the pancreas to fatty acids may also lead to the inhibition of insulin secretion and the development of type 2 diabetes (41). However, in various rodent knockout models, plasma fatty acid levels have not always correlated with changes in insulin sensitivity (42).

Furthermore, the absence of adipose tissue in mouse models of lipodystrophy results in severe insulin resistance and diabetes (43,44). Although adipose tissue was classically considered a passive depot for energy storage and release, adipocytes have recently been shown to express and secrete several peptide hormones and cytokines, such as tumor necrosis factor alpha (TNFα), leptin, adiponectin (ACRP30), interleukin-6 and resistin (45). The ability of these hormones and cytokines to influence glucose and fatty acid metabolism has expanded the role of the adipocyte in insulin resistance and suggested that fatty acids are most likely not the only factor involved in the development of insulin resistance. The potential role of leptin and adiponectin in the regulation of glucose and fatty acid metabolism will be discussed in a later section.

Role of Substrate Competition in the Development of Insulin Resistance

Altered fatty acid and glucose metabolism play a key role in the development of insulin resistance. Alterations in insulin action play an important role in mediating these changes in metabolism, since insulin controls the balance between fuel production and fuel use through its actions on skeletal muscle, heart, liver and adipose tissue. However, skeletal muscle is the primary tissue responsible for glucose disposal in response to insulin (46) and is largely responsible for peripheral insulin resistance (47).

The glucose-fatty acid cycle, first proposed by Randle in 1963, states that as fatty acid availability increases, muscle fatty acid oxidation will increase resulting in a reciprocal reduction in glucose metabolism including glucose uptake, glycolysis and glucose oxidation (38). The key points of the Randle cycle are elevations in acetyl-CoA/CoA levels from fatty acid oxidation leading to inhibition of the PDH complex. Increased citrate levels are negative modulators of PFK-1 leading to reductions in glycolysis. Blockade of PFK-1 results in an accumulation of glucose-6-phosphate which inhibits hexokinase activity and reduces glucose uptake. While indirect calorimetry studies in type 2 diabetic patients have consistently demonstrated that fatty acid infusion reduces whole body glucose utilization (39,40), studies in insulin resistant skeletal muscle have provided inconsistent data. While some studies have demonstrated increased fatty acid oxidation (48), others have demonstrated decreased fatty acid oxidation (49-51) and yet others show no significant change in fatty acid oxidation (52). Thus, it is not yet clear whether the glucose-fatty acid cycle plays a role in the development of insulin resistance and there is still controversy as to whether muscle fatty acid oxidation is altered.

Role of Triacylglycerol Accumulation in the Development of Insulin Resistance

Insulin resistance is characterized by the accumulation of intracellular TG in muscle. This accumulation of intracellular TG or 'steatosis' in muscle is highly correlated with the development of insulin resistance, as well as the severity of insulin resistance [reviewed in 53]. In support of this, reduction of skeletal muscle TG, with either troglitazone or leptin, results in an increase in insulin-stimulated glucose uptake and a decrease in insulin resistance (39,54). Furthermore, the inactivation of enzymes involved in fatty acid uptake and storage (LPL, DGAT) is associated with increases in insulin sensitivity [reviewed in 42]. In contrast, the overexpression of these enzymes results in TG accumulation and reductions in insulin sensitivity (42).

Although there is a strong correlation between TG accumulation and insulin resistance, the accumulation of TG itself is not detrimental to muscle insulin sensitivity. However, the accumulation of TG in muscle is believed to increase the availability of intracellular lipid products such as long-chain acyl-CoAs and DG [reviewed in 55]. In skeletal muscle, this 'lipotoxic' effect of TG accumulation can lead to impaired insulin signaling and the development of insulin resistance (56-58). For example, intermediates in TG synthesis such as long chain acyl-CoA and DG can activate isoforms of the novel protein kinase C family (δ,ε,θ) which have been demonstrated to interfere with insulin signaling [reviewed in 59]. Furthermore, long chain acyl-CoAs can activate the transcription factor, peroxisome proliferator activated receptor alpha (PPARα), involved in the transcriptional control of regulatory enzymes in fatty acid metabolism [reviewed in 59]. While several mechanisms for an inhibitory role of long chain acyl-CoAs on insulin signaling have been proposed, the precise mechanisms remain unclear.

While the metabolic capacity of insulin resistant muscle appears to be organized toward TG storage rather than fatty acid oxidation [reviewed in 60], it remains controversial whether the accumulation of TG in muscle is the result of increased fatty acid supply, decreased fatty acid oxidation, or a combination of both. It is also not clear whether increases in TG synthesis or decreases in TG lipolysis play a role in the accumulation of muscle TG. However, studies have shown that the inhibition of muscle fatty acid oxidation results in TG accumulation and insulin resistance (61,62). Recently, Kelley and Mandarino have re-examined the current data on fatty acid oxidation in insulin resistant muscle (60). They have proposed that the development of insulin resistance results due to 'metabolic inflexibility' rather than absolute changes in fatty acid oxidation (60). According to this theory, the insulin resistant muscle fails to augment fatty acid oxidation during fasting or reduce fatty acid oxidation after a meal (60). Thus, fatty acids are not removed appropriately during different nutritional states and result in the accumulation of intracellular TG (60).

Alternatively, the controversy regarding alterations in fatty acid oxidation may be explained by considering that different alterations in fatty acid metabolism occur during the development of insulin resistance [reviewed in 63]. It has been proposed that elevated fatty acids initially stimulate fatty acid oxidation resulting in elevations in acetyl-CoA. Elevated acetyl-CoA through mechanisms described in the Randle cycle, inhibits glucose utilization resulting in insulin resistance. Insulin resistance in the periphery is automatically compensated for by hypersecretion of insulin. High levels of insulin are able to stimulate glucose utilization producing acetyl-CoA that inhibits fatty acid oxidation according to the reverse glucose-fatty acid cycle (64). The inhibition of fatty acid oxidation results in the accumulation of TG and long-chain acyl-CoAs which maintain insulin resistance. Thus, elevated fatty acids may not result in increases in fatty acid oxidation due to the inhibitory effect of hyperinsulinemia on fatty acid oxidation. Although this mechanism has not been proven directly, it highlights the importance of identifying the stage of insulin resistance that is being studied.

Coordinated Regulation of Fatty Acid Metabolism

Both fatty acid synthesis and fatty acid oxidation are controlled by acetyl-CoA carboxylase (ACC). This dual regulatory role of ACC is accomplished by the existence of two isoforms (65). The 265 kDa isoform of ACC (ACC-265) is the rate-limiting enzyme for fatty acid biosynthesis in liver and adipose tissue (66). The 280 kDa isoform of ACC (ACC-280) is a major regulator of muscle fatty acid metabolism (67-69), since malonyl-CoA produced by this enzyme is a potent inhibitor of carnitine palmitoyltransferase-I (CPT-I), a key enzyme involved in mitochondrial fatty acid uptake (70).

Both isoforms of ACC can be regulated at the level of gene expression, allosteric regulation of the enzyme, and reversible phosphorylation by several kinases (71,72). In liver, skeletal muscle and heart, AMP-activated protein kinase (AMPK) phosphorylates ACC, and contributes significantly to the regulation of ACC activity (73-75).

Coordinated Regulation of Fatty Acid and Glucose Metabolism

The AMPK cascade acts as a metabolic sensor or "fuel gauge" in the mammalian cell [reviewed in 76]. This role is fulfilled by the ability of AMPK to phosphorylate key target proteins that control flux through metabolic pathways of glucose uptake, glycolysis, gluconeogenesis, fatty acid synthesis, cholesterol synthesis, fatty acid oxidation, TG synthesis and TG lipolysis (76-79). The role of AMPK in the regulation of fatty acid and glucose metabolism is depicted in Figure

1-3. AMPK can be activated through phosphorylation on threonine 172 by its upstream kinase, AMPK kinase (AMPKK) (80) and through increases in adenosine monophosphate: adenosine triphosphate (AMP:ATP) (81) and creatine:phosphocreatine (Cr:PCr) ratios (82).

AMPK is a heterotrimeric complex comprising an α catalytic subunit and two regulatory subunits (β and γ) (83). Several isoforms of each subunit have been identified with varying tissue distribution (84). At least 2 isoforms of the α catalytic subunit have been identified: the $\alpha 2$ isoform represents the majority of AMPK activity in muscle, while the $\alpha 1$ isoform predominates in liver (84).

Through the coordinated regulation of key enzymes in fatty acid metabolism, AMPK regulates the flux of fatty acids into oxidation rather than TG storage (79). AMPK activation increases fatty acid oxidation by inhibiting ACC and reducing malonyl-CoA levels (74,75). The activation of AMPK also results in the phosphorylation and inhibition of the mitochondrial glycerol-3-phosphate acyltransferase (GPAT), the first committed step in *de novo* synthesis of TG (79).

AMPK plays a key role in the regulation of glucose metabolism. In the heart, the activation of AMPK results in a stimulation of glucose uptake via GLUT-4 translocation (77). AMPK also phosphorylates phosphofructokinase-2 (PFK-2) resulting in a stimulation of glycolysis in the heart (78). In liver, AMPK phosphorylation of PFK-2 also stimulates glycolysis, resulting in a reduction in hepatic glucose production (85).

There is currently controversy about the role of AMPK in the regulation of glycogen metabolism. AMPK has been shown to phosphorylate glycogen synthase and phosphorylase kinase *in vitro* (86). However, the activation of AMPK with AICAR stimulates glycogen phosphorylase activity and glycogen breakdown in some studies (87), increases glycogen content through activation of glycogen synthase in others (88), and increases glycogen content with no effect on glycogen synthase or phosphorylase activities in yet other studies (89). Recent studies have demonstrated that the amount of glycogen present may influence the AICAR-induced activation of AMPK suggesting that AMPK may be regulated by glycogen stores (89,90). Nevertheless, in both muscle and heart, activating mutations in $\gamma 3$ or $\gamma 2$ subunits of AMPK, respectively, result in glycogen storage disease (91,92). Although the mutations are thought to result in AMPK activation, recent studies have suggested that these mutations may not be activating (93). Furthermore, there is presently little information about the roles of the different AMPK heterotrimers and whether specific isoforms are responsible for the control of fatty acid or glucose metabolism.

Consistent, with the role of AMPK as a cellular energy gauge (47), AMPK has been shown to be regulated by hormones and energy substrates. For instance, AMPK is inhibited by insulin in heart and liver (94-96) and by high glucose in pancreatic beta cells (97). Furthermore, acute exposure to fatty acids stimulates AMPK activity while chronic exposure to fatty acids significantly reduces AMPK activity in neonatal cardiomyocytes (98). These data suggest that the hyperinsulinemia and hyperlipidemia characteristic of insulin resistance could lead to the downregulation of AMPK.

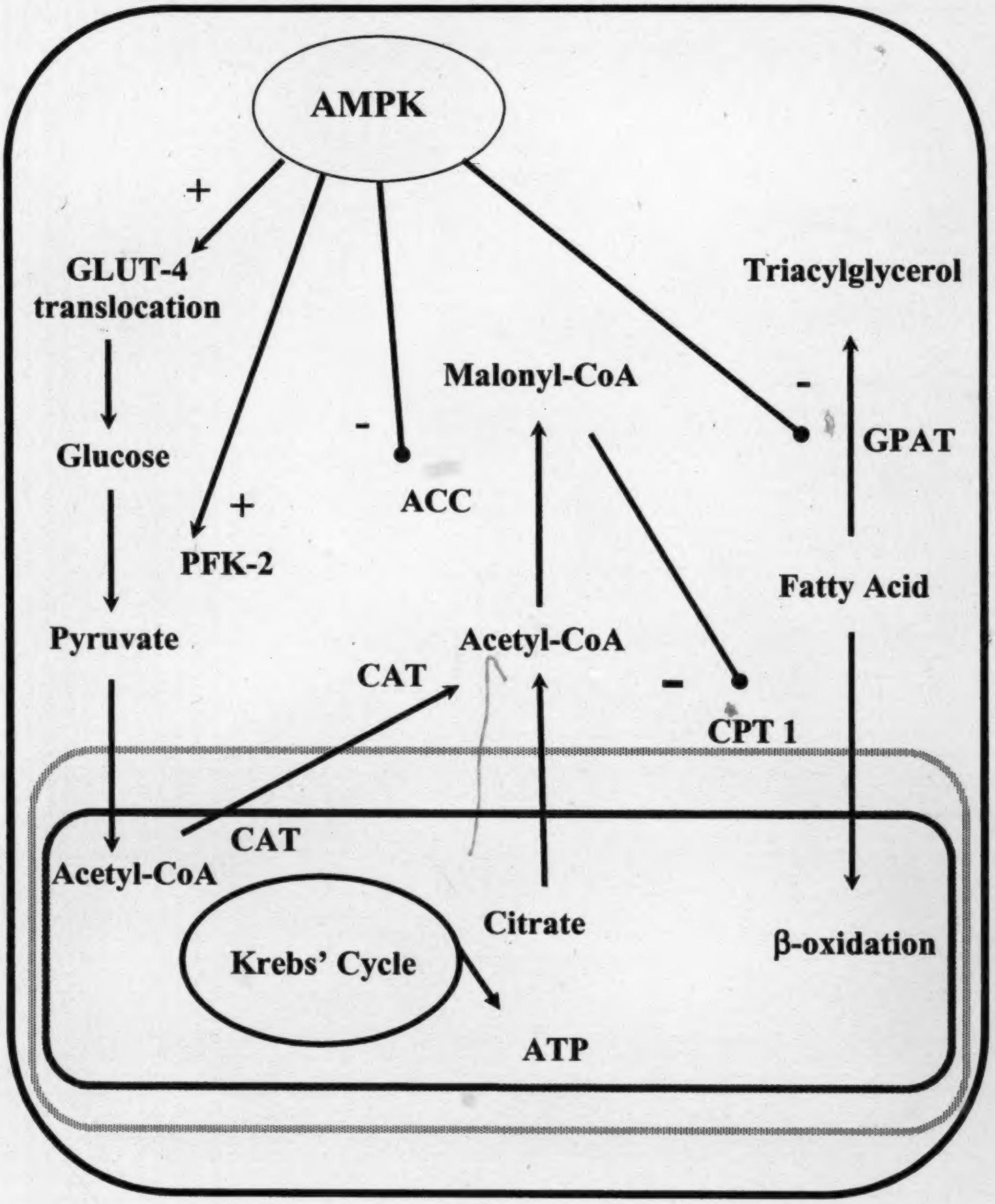

AMPK
+
GLUT-4 translocation
Glucose
Pyruvate
Acetyl-CoA
+
PFK-2
-
ACC
Malonyl-CoA
Acetyl-CoA
CAT
CAT
-
CPT 1
Citrate
Krebs' Cycle
ATP
Triacylglycerol
-
GPAT
Fatty Acid
β-oxidation

Figure 1-3. Role of AMPK in the regulation of fatty acid and glucose metabolism.

Hormonal Regulation of Fatty Acid Metabolism

Leptin

Leptin is a peptide hormone synthesized by adipocytes (99) that plays a key role in the regulation of appetite and energy expenditure through its actions in the hypothalamus (100). Accumulating evidence now suggests that leptin also regulates energy homeostasis through direct actions on peripheral lipid and glucose metabolism (101).

The key role of leptin is demonstrated in mice with mutations in the gene encoding either the leptin protein (ob/ob) or leptin receptor (db/db). These mice exhibit an insulin resistant syndrome characterized by obesity, hyperinsulinemia and hyperlipidemia (102-103). Leptin treatment of ob/ob mice results in reduced food intake, increased energy expenditure and alleviation of the insulin resistant syndrome (102-103).

Leptin mediates its actions through leptin receptors expressed in the hypothalamus, liver, heart, kidney, small intestine, testis, adipose tissue and spleen (104). The leptin receptor is a member of the class I cytokine receptor family (105). Alternative splicing of the leptin receptor gene results in six isoforms that differ only in the intracellular domain (106,107). The long isoform (OB-Rb) has a long intracellular domain and signals through the JAK (janus kinase) - STAT (signal transducers and activators of transcription) pathway (108-111). The other isoforms (OB-Ra, OB-Rc, OB-Rd, OB-Rf) have shorter intracellular domains with unknown signaling pathways. OB-Re lacks the transmembrane domain and is believed to be a soluble leptin receptor (106).

In liver, skeletal muscle and pancreas, leptin partitions fatty acids toward fatty acid oxidation rather than TG storage. For instance, in vivo elevation of leptin levels in normal rats leads to a depletion of TG content in liver, skeletal muscle and pancreas without an increase in plasma fatty acids or ketones, suggesting intracellular oxidation (54). Furthermore, acute and chronic leptin treatment results in increased fatty acid oxidation and decreased incorporation of fatty acids into TG (112-114). In rodent models of leptin deficiency or leptin inaction, the accumulation of TG is prominent in skeletal muscle, heart, liver and pancreas (56).

Roger Unger has proposed that the main physiological role of leptin is to protect tissues other than adipose tissue from TG accumulation [reviewed in 56]. Indeed, leptin deficiency or leptin receptor mutations result in a significant accumulation of TG in non-adipose tissues (56). Furthermore, mice in which DGAT-1 is overexpressed in adipose tissue results in a significant increase in obesity with normal insulin sensitivity and normal levels of hepatic and skeletal muscle TG (115). While this may result due to the increased storage of fatty acids in adipose tissue and reduced fatty acid flux to liver and muscle, elevated plasma leptin levels may be responsible for the lack of TG accumulation. Further support for a role of leptin in the maintenance of tissue TG levels comes from mice lacking adipose tissue. Overexpression of leptin in these mice restores tissue TG levels and improves insulin resistance (116). Furthermore, the transplantation of adipose tissue lacking leptin into adipose deficient mice is unable to reverse the insulin resistance suggesting that leptin plays a key role in peripheral insulin sensitivity through the maintenance of tissue TG levels (117).

The mechanism by which leptin increases fatty acid oxidation and decreases TG content in peripheral tissues is not completely understood. Very recently, leptin was suggested to increase fatty acid oxidation acutely in skeletal muscle through the activation of AMP-activated protein kinase (AMPK) (118).

Adiponectin

Adiponectin is another adipose-derived hormone that has been demonstrated to play a role in obesity and insulin resistance. Unlike leptin, adiponectin levels are reduced in obesity and insulin resistance (119). Adiponectin has been shown to increase muscle fatty acid oxidation through AMPK activation and reduce tissue TG levels leading to improvements in insulin sensitivity (120-122). However, it is not clear why both leptin and adiponectin activate AMPK and increase fatty acid oxidation since leptin levels are characteristically elevated and adiponectin levels are reduced in obesity and insulin resistance. It is also unclear why mice lacking adiponectin have elevated rates of fatty acid oxidation (123). The identification of the adiponectin receptor(s) and tissue localization will provide more clues about the physiological and pathophysiological roles of adiponectin.

Cardiac Metabolism and Insulin Resistance

The metabolism of energy substrates is crucial to maintain contractile function in the heart. Under normal physiological conditions, the energy requirements of the heart are fulfilled primarily by the oxidation of fatty acids with the remainder provided by carbohydrate metabolism (124). Several studies have implicated abnormal fatty acid metabolism as being an important contributor to the pathogenesis of diabetes-related cardiovascular dysfunction (125-128).

In type 1 diabetes, elevated plasma fatty acids result in a decreased utilization of glucose and an over-reliance of the heart on fatty acid oxidation [reviewed in 125]. Elevated rates of fatty acid oxidation are associated with increased AMPK activity, and decreased ACC activity and malonyl-CoA levels. Furthermore, insulin treatment restores AMPK activity, ACC activity, malonyl-CoA levels and fatty acid oxidation rates to normal levels (95). Similarly, increased rates of cardiac fatty acid oxidation have been demonstrated in db/db mice, a model of type 2 diabetes (126). In both type 1 and type 2 diabetes, alterations in fatty acid oxidation are correlated with changes in contractile function (125-127).

TG accumulation has been identified as an important contributor to the pathogenesis of diabetes-related cardiovascular dysfunction. The accumulation of cardiac TG is associated with depressed contractility, arrhythmias, hypertrophy, heart failure and apoptosis (128-133). Importantly, the reduction of cardiac TG content with troglitazone is associated with improvements in contractile dysfunction (129) suggesting that TG accumulation may play a role in diabetes-related cardiovascular dysfunction.

The presence of cardiac insulin resistance in hypertensive (22) and aged rats (23), as well as in patients with angina (24), heart failure and coronary heart disease (25) has demonstrated a link between insulin resistance and cardiovascular disease. However, it is not clear whether the heart becomes resistant to the effects of insulin or whether alterations in fatty acid metabolism contribute to insulin resistance (25,26).

Experimental Models of Insulin Resistance

Several dietary and genetic models of insulin resistance exist [reviewed in 134]. The high-fat fed rat is a commonly used model of insulin resistance. Since high fat feeding of normal Sprague Dawley rats induces obesity and insulin resistance, this model is thought to parallel obesity and insulin resistance induced by overeating in humans (135). The ob/ob mouse is a genetic model of insulin resistance due to a mutation in the gene encoding the leptin protein (ob). The db/db mouse has a mutation resulting in the absence of the long form of the leptin receptor (OB-Rb). These mice exhibit an insulin resistant syndrome characterized by obesity, hyperinsulinemia and hyperlipidemia (102-104).

The Zucker fatty rat (fa/fa) is another model of leptin receptor deficiency. However, the fa mutation is a missense mutation that results in reduced signaling ability of the leptin receptor (136). The obese Zucker rat may develop hyperglycemia and type 2 diabetes depending on the background strain of the rat (134).

The JCR:LA-cp rat is closely related to the Zucker fatty rat. The JCR:LA-cp rat is a model of pre-type 2 diabetes that displays complete skeletal muscle insulin resistance by the age of 12 weeks (137). The JCR:LA-cp rat is deficient in the leptin receptor due to a mutation encoded by the cp gene (138). The development of insulin resistance occurs between the age of 4 and 12 weeks and is characterized by obesity, hyperinsulinemia, and hyperlipidemia (137). The JCR:LA-cp is a good model of insulin resistance because it does not progress to type 2 diabetes, unlike other rodent models. Furthermore, the JCR:LA-cp rat closely resembles the human metabolic syndrome because it develops spontaneous cardiovascular disease similar to that seen in humans (139).

The accumulation of TG in muscle of the JCR:LA-cp rat is strongly associated with the onset of muscle insulin resistance. Both electron microscopy and oil red O staining have confirmed the presence of intracellular lipid droplets in the muscle of 12 week JCR:LA-cp rats (137). Elevated fatty acid supply has been demonstrated to play a role in the accumulation of muscle TG in the JCR:LA-cp rat (137), however, it is not clear whether alterations in muscle fatty acid metabolism also contribute to the accumulation of muscle TG in the JCR:LA-cp rat.

Modification of the Insulin Resistant Syndrome

The development of insulin resistance involves alterations in fatty acid and glucose metabolism suggesting that enzymes involved in the regulation of fatty acid and glucose metabolism may represent novel targets for the treatment of insulin resistance. The lipid-lowering agent, MEDICA 16, is an ATP:citrate lyase inhibitor that limits acetyl-CoA supply to ACC, resulting in a reduction in ACC activity (140). Indeed, chronic treatment with MEDICA 16 reduces plasma TG concentrations by 80% and blunts the development of insulin resistance in JCR:LA-cp rats (137), as well as in the obese Zucker rat (141). Acutely, MEDICA 16 treatment results in a significant decrease in plasma VLDL cholesterol and TG (142) and a significant inhibition of hepatic lipogenesis (143). However, the demonstration that mice lacking ACC-280 have higher rates of muscle fatty acid oxidation and significantly reduced amounts of fat (144) has lead to the suggestion that activators of fatty acid oxidation may also be promising new drugs in the prevention of obesity.

AMPK is considered a novel therapeutic target for the treatment of obesity, insulin resistance and type 2 diabetes (145-147). Recently, AMPK has been identified as a target of the insulin sensitizing agents, metformin, troglitazone and rosiglitazone (147-151). Consistent with this role, treatment of insulin resistant rats with AICAR, a non-specific AMPK activator, results in an improvement in insulin action (152) and AICAR treatment of type 2 diabetic rodents results in a significant reduction in plasma glucose levels (153-157).

On the other hand, recent studies have described activating AMPK mutations which result in functional abnormalities and hypertrophy in the heart (92,158-160). For instance, mutations in the $\gamma 2$ subunit of AMPK result in glycogen storage disease that mimics hypertrophic cardiomyopathy (92). Furthermore, mutations in the $\gamma 2$ subunit of AMPK cause ventricular preexcitation, atrial fibrillation, conduction defects and cardiac hypertrophy (158-160). Studies have also demonstrated elevated AMPK activity in pressure overload hypertrophy (161). However, it is currently controversial whether these mutations result in AMPK activation (93,162). Thus, although the activation of AMPK may be beneficial in correcting the abnormal glucose and fatty acid metabolism accompanying insulin resistance, AMPK activation may result in cardiac hypertrophy and functional abnormalities. Clearly the role of AMPK in the regulation of cellular energy balance is complex and requires intense investigation before AMPK can become the target for insulin-sensitizing agents.

Exercise Training and Insulin Sensitivity

The ability of exercise training to improve insulin sensitivity in insulin resistant patients or rodents is well characterized [reviewed in 163]. The observation that insulin and contraction stimulate GLUT4 translocation through distinct signaling pathways has provided a potential explanation for the beneficial effects of exercise training on insulin sensitivity [reviewed in 164]. Muscle contraction, by electrical stimulation or exercise, activates AMPK and GLUT4 translocation independent of the insulin signaling pathway [reviewed in 165,166]. The ability of exercise to activate AMPK in insulin resistant human muscle has provided further evidence that AMPK may mediate the exercise-induced improvements in insulin sensitivity (167,168). This notion has generated tremendous interest in the development of AMPK activators for the treatment of insulin resistance.

Models of Exercise Training

The use of exercise training in rodents has yielded conflicting data in the past. While treadmill running, motorized exercise wheels and forced swimming are used routinely, there is evidence that these models evoke a stress response in rodents (169). An alternative model of exercise training is voluntary wheel running induced by mild food restriction (170). This model has several benefits compared with other exercise training models. For example, mild food restriction does not affect the physiological response of rodents to exercise-training (169). Furthermore, voluntary wheel running offers a more relevant model to the human condition because exercise in humans is usually accompanied by a reduction in food intake (169). This model of exercise-training has been characterized in the JCR:LA-cp rat (170). Voluntary wheel running in these animals is highly correlated with food intake and can be easily manipulated to obtain a certain level of exercise training (170).

Hypothesis and Objectives

AMPK plays a key role in the regulation of fatty acid and glucose metabolism. Not only would the hyperinsulinemia and hyperlipidemia characteristic of the insulin resistant syndrome be expected to reduce AMPK activity, a reduction in AMPK activity could account for reduced glucose metabolism, decreased fatty acid oxidation and elevated TG content. Thus, we hypothesized that a reduction in AMPK activity is associated with altered fatty acid and glucose metabolism in the insulin resistant JCR:LA-cp rat heart.

Leptin regulates fatty acid metabolism in liver, skeletal muscle and pancreas by partitioning fatty acids into oxidation rather than TG storage. Since leptin receptors are present in the heart, we investigated whether leptin directly regulates cardiac fatty acid oxidation and TG content in isolated working rat hearts. We further investigated whether leptin action was associated with the activation of AMPK.

The insulin resistant JCR:LA-cp rat is leptin receptor deficient due to a mutation in the leptin receptor gene. Thus, we investigated whether the absence of leptin signaling results in altered cardiac fatty acid oxidation and TG content in isolated working JCR:LA-cp rat hearts. We also investigated whether the accumulation of TG in the heart is associated with reduced glucose utilization. We further investigated whether changes in AMPK activity are associated with altered fatty acid and glucose metabolism in the insulin resistant hearts.

The accumulation of cardiac TG can be the result of increased fatty acid supply, decreased fatty acid oxidation, increased TG synthesis or decreased TG lipolysis, or a combination of these. Our data suggests that increased fatty acid supply rather than reduced fatty acid oxidation or increased TG synthesis is contributing to TG accumulation in the JCR:LA-cp rat heart. Thus, we extended our studies to investigate potential alterations in ACC and AMPK in the liver and skeletal muscle of the JCR:LA-cp rat during the development of insulin resistance. We also treated JCR:LA-cp rats with the lipid-lowering compound, MEDICA 16 to investigate further the role of ACC and AMPK in the insulin resistant syndrome.

The activation of AMPK is considered a novel target in the treatment of obesity and insulin resistance. Exercise improves insulin sensitivity and activates AMPK. We determined whether exercise can activate AMPK in the heart and whether the exercise-induced activation of AMPK was associated with altered cardiac fatty acid oxidation, TG content and glycolysis.

References

1. James PT, Leach R, Kalamara E, Shayeghi M: The worldwide obesity epidemic. *Obes Res* 9(Suppl 4):228S-233S, 2001
2. Katzmarzyk PT: The Canadian obesity epidemic: an historical perspective. *Obes Res* 10:666-674, 2002
3. Flegal KM, Carroll MD, Ogden DL, Johnson CL: Prevalence and trends in obesity among US adults, 1999-2000. *JAMA* 288:1723-1727, 2002
4. Felber JP, Golay A: Pathways from obesity to diabetes. *Int J Obes Relat Metab Disord* 26(Suppl 2):S39-S45, 2002
5. Harris MI, Flegal KM, Cowie CC, Eberhardt MS, Goldstein DE, Little RR, Wiedmeyer HM, Byrd-Holt, DD: Prevalence of diabetes, impaired fasting glucose and impaired glucose tolerance in US adults. The third national health and nutrition examination survey, 1988-1994. *Diabetes Care* 21:518-524, 1998
6. Flegal KM, Carroll M, Ogden CL, Johnson CL: Prevalence and trends in obesity among US adults, 1999-2000. *JAMA* 2[illegible]8:1723-1727, 2002
7. Ogden CL, Flegal KM, Carroll MD, Johnson CL: Prevalence and trends in overweight among US children and adolescents, 1999-2000. *JAMA* 288:1728-1732, 2002
8. Reaven GM: Role of insulin resistance in human disease (Syndrome X): an expanded definition. *Annu Rev Med* 44:121-131, 1993

9. Lebovitz HE: Insulin resistance: definition and consequences. *Exp Clin Endocrinol Diabetes* 109(Suppl 2):S135-S148, 2001

10. Alberti K, Zimmet P, Consultation W: Definition diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus, provisional report of a WHO consultation. *Diabetes Med* 15:539-553, 1998

11. Olefsky JM, Ciaraldi TP, Kolterman OG: Mechanisms of insulin resistance in non-insulin-dependent (type II) diabetes. *Am J Med* 79:12-22, 1985

12. Hall JE, Crook ED, Jones DW, Wofford MR, Dubbert PM: Mechanisms of obesity-associated cardiovascular and renal disease. *Am J Med Sci* 324:127-137, 2002

13. Hubert HB, Feinleib M, McNamara PM, Castelli WP: Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framington Study. *Circulation* 67:968-977, 1983

14. Rao SV, Donahue M, Pi-Sunyer FX, Fuster V: Obesity as a risk factor in coronary artery disease. *Am Heart J* 142: 1102-1107, 2001

15. Vega GL: Obesity, the metabolic syndrome, and cardiovascular disease. *Am Heart J* 142: 1108-1116, 2001

16. Manson JE, Tosteson H, Ridker PM, Satterfield S, Hebert P, O'Connor GT, Buring JE, Hennekens CH: The primary prevention of myocardial infarction. *N Engl J Med* 326:1406-1416, 1992

17. Abbasi F, Brown BW Jr, Lamendola C, McLaughlin T, Reaven GM: Relationship between obesity, insulin resistance, and coronary heart disease risk. *J Am Coll Cardiol* 40:937-943, 2002

18. Taegtmeyer H: Insulin resistance and atherosclerosis: common roots for two common diseases. *Circulation* 93:1777-1779, 1996

19. Ginsberg HN: Insulin resistance and cardiovascular disease. *J Clin Invest* 106:453-458, 2000

20. Yip J, Facchini FS, Reaven GM. Resistance to insulin-mediated glucose disposal as a predictor of cardiovascular disease. *J Clin Endocrinol Metab* 83: 2773-2776, 1998

21. Reaven GM: Pathophysiology of Insulin Resistance in Human Disease. *Physiol Rev* 75:473-486, 1985

22. Morisco C, Condorelli G, Orzi F, Vigliotta G, Di Grezia R, Beguinot F, Trimarco B, Lembo G: Insulin-stimulated cardiac glucose uptake is impaired in spontaneously hypertensive rats: role of early steps of insulin signaling. *J Hypertens* 18:465-473, 2000

23. Escriva F, Agote M, Rubio E, Molero JC, Pascual-Leone AM, Andres A, Satrustegui J, Carrascosa JM: In vivo insulin-dependent glucose uptake of specific tissues is decreased during aging of mature Wistar rats. *Endocrinology* 138:49-54, 1997

24. Botker HE, Moller N, Schmitz O, Bagger JP, Nielsen TT: Myocardial insulin resistance in patients with syndrome X. *J Clin Invest* 100:1919-1927, 1997

25. Paternostro G, Camici PG, Lammerstma AA, Marinho N, Baliga RR, Kooner JS, Radda GK, Ferrannini E: Cardiac and skeletal muscle insulin resistance in patients with coronary heart disease. A study with positron emission tomography. *J Clin Invest* 98:2094-2099, 1996

26. Iozzo G, Chareonthaitawee P, Dutka D, Betteridge DJ, Ferrannini E, Camici PG: Independent association of type 2 diabetes and coronary artery disease with myocardial insulin resistance. *Diabetes* 51:3020-3024, 2002

27. Glatz JF, Luiken JJ, Bonen A: Involvement of membrane-associated proteins in the acute regulation of cellular fatty acid uptake. *J Mol Neurosci* 16:123-132, 2001

28. West DW, Chase JF, Tubbs PK: The separation and properties of two forms of carnitine palmitoyltransferase from ox liver mitochondria. *Biochem Biophys Res Commun* 42:912-918, 1971

29. McGarry JD, Stark MJ, Foster DW: Hepatic malonyl-CoA levels of fed, fasted and diabetic rats as measured using a simple radioisotopic assay. *J Biol Chem* 253:8291-8293, 1978

30. Somwar R, Kim DY, Sweeney G, Huang C, Niu W, Lador C, Ramlal T, Klip A: GLUT4 translocation precedes the stimulation of glucose uptake by insulin in muscle cells: potential activation of GLUT4 via p38 mitogen-activated protein kinase. *Biochem J* 359:639-649, 2001

31. Bogardus C, Lillioja S, Mott DM, Hollenbeck C, Reaven G: Relationship between degree of obesity and in vivo insulin action in man. *Am J Physiol* 248:E286-E291, 1985

32. Boden G: Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes* 46:3-10, 1997

33. Gordon ES: Non-esterified fatty acids in blood of obese and lean subjects. *Am J Clin Nutr 8*:740-747, 1960

34. Reaven GM, Hollenbeck C, Jeng CY, Wu MS, Chen YD: Measurement of plasma glucose, free fatty acid, lactate and insulin for 24 h in patients with NIDDM. *Diabetes* 37:1020-1024, 1988

35. Bjorntorp P, Bergman H, Varnauskas E: Plasma free fatty acid turnover in obesity. *Acta Med Scand* 185:351-356, 1969

36. Jensen MD, Haymond MW, Rizza RA, Cryer PE, Miles JM: Influence of body fat distribution on free fatty acid metabolism in obesity. *J Clin Invest* 83:1168-1173, 1989

37. Ferrannini E, Barrett EJ, Bevilacqua S, DeFronzo RA: Effect of fatty acids on glucose production and utilization in man. *J Clin Invest* 72:1737-1747, 1983

38. Randle PJ, Garland PB, Hales CN, Newsholme EA: The glucose fatty-acid cycle: its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* i:785-789, 1963

39. Roden M, Price TB, Perseghin G, Falk Petersen K, Rothman DL, Cline GW, Shulman GI: Mechanism of free fatty acid-induced insulin resistance in humans. *J Clin Invest* 97:2859-2865, 1996

40. Kelley DE, Mokan M, Simoneau JA, Mandarino LJ: Interaction between glucose and free fatty acid metabolism in human skeletal muscle. *J Clin Invest* 92:91-98, 1993

41. Boden G: Interaction between free fatty acids and glucose metabolism. *Curr Opin Clin Nutr Metab* Care 5:545-549, 2002

42. Chen HC, Farese RV Jr: Fatty acids, triglycerides, and glucose metabolism: recent insights from knockout mice. *Curr Opin Clin Nutr Metab Care* 5:359-363, 2002

43. Moitra J, Mason MM, Olive M, Krylov D, Gavrilova O, Marcus-Samuels B, Feigenbaum L, Lee E, Aoyama T, Eckhaus M, Reitman ML, Vinson C: Life without white fat: a transgenic mouse. *Genes Dev* 12:3168-3181, 1998

44. Shimomura I, Hammer RE, Richardson JA, Ikemoto S, Bashmakov Y, Goldstein JL, Brown MS: Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: a model for congenital generalized lipodystrophy. *Genes Dev* 12:3182-3194, 1998

45. Mora S, Pessin JE: An adipocentric view of signaling and intracellular trafficking. *Diabetes Metab Res Rev* 18:345-356, 2002

46. DeFronzo RA, Jocot E, Jecquier E, Maeder E, Wahren J, Felber JP: The effect of insulin on the disposal of intravenous glucose. *Diabetes* 30:1000-1007, 1981

47. DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, Wahren J: Effects of insulin on peripheral and splanchnic glucose metabolism in non-insulin-dependent (type II) diabetes mellitus. *J Clin Invest* 76:149-155, 1985

48. Sreenan S, Keck S, Fuller T, Cockburn B, Burant CF: Effects of troglitazone on substrate storage and utilization in insulin-resistant rats. *Am J Physiol* 276:E1119-E1129, 1999

49. Ide T, Nakazawa T, Mochizuki T, Murakami K: Tissue-specific actions of antidiabetic thiazolidinediones on the reduced fatty acid oxidation in skeletal muscle and liver of Zucker diabetic fatty rats. *Metabolism* 49:521-525, 2000

50. Kelley DE, Simoneau J-A: Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus. *J Clin Invest* 94:2349-2356, 1994

51. Kim J-Y, Hickner RC, Cortright RL, Dohm GL, Houmard JA: Lipid oxidation is reduced in obese human skeletal muscle. *Am J Physiol* 279:E1039-E1044, 2000

52. Turcotte LP, Swenberger JR, Tucker MZ, Yee AJ: Increased fatty acid uptake and altered fatty acid metabolism in insulin-resistant muscle of obese Zucker rats. *Diabetes* 50:1389-1396, 2001

53. Lewis GF, Carpentier A, Khosrow A, Giacca A: Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. *Endocrine Rev* 23:201-229, 2002

54. Shimabukuro M, Koyama K, Chen G, Wang MY, Trieu F, Lee Y, Newgard CB, Unger RH: Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc Natl Acad Sci USA* 94:4637-4641, 1997

55. Kraegen EW, Cooney GJ, Ye J, Thompson AL: Triglycerides, fatty acids and insulin resistance—hyperinsulinemia. *Exp Clin Endocrinol Diabetes* 109:S516-S526, 2001

56. Unger RH: Lipotoxic disease. *Annu Rev Med* 53:319-336, 2002

57. Virkamaki A, Korsheninnikova E, Seppala-Lindroos A, Vehkavaara S, Goto T, Halavaara J, Hakkinen AM, Yki-Jarvinen H: Intramyocellular lipid is associated with resistance to in vivo insulin actions on glucose uptake, antilipolysis, and early insulin signaling pathways in human skeletal muscle. *Diabetes* 50:2337-2343, 2001

58. Kraegen EW, Cooney GJ, Ye JM, Thompson AL, Furler SM: The role of lipids in the pathogenesis of muscle insulin resistance and beta cell failure in type II diabetes and obesity. *Exp Clin Endocrinol Diabetes* 109:S189-S201, 2001

59. Cooney GJ, Thompson AL, Furler SM, Ye J, Kraegen EW: Muscle long-chain acyl CoA esters and insulin resistance. *Ann NY Acad Sci* 967:196-207, 2002

60. Kelley DE, Mandarino LJ: Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes* 49:677-683, 2000

61. Dobbins RL, Szczepaniak LS, Bentley B, Esser V, Myhill J, McGarry JD: Prolonged inhibition of muscle carnitine palmitoyltransferase-1 promotes intramyocellular lipid accumulation and insulin resistance in rats. *Diabetes* 50:123-130, 2001

62. Hegarty BD, Cooney GJ, Kraegen EW, Furler SM: Increased efficiency of fatty acid uptake contributes to lipid accumulation in skeletal muscle of high fat-fed insulin-resistant rats. *Diabetes* 51:1477-1484, 2002

63. Belfiore F, Iannello S: Insulin resistance in obesity: metabolic mechanisms and measurement methods. *Mol Genet Metab* 65:121-128, 1998

64. Wolfe RR: Metabolic interactions between glucose and fatty acids in humans. *Am J Clin Nutr* 67:519S-526S, 1998

65. Munday MR, Hemingway CJ: The regulation of acetyl-CoA carboxylase: a potential target for the action of hypolipidemic agents. *Advan Enzyme Regul* 39:205-234, 1999

66. Goodridge AG: Regulation of fatty acid synthesis in isolated hepatocytes: evidence for a physiological role for long chain fatty acyl coenzyme A and citrate. *J Biol Chem* 248:4318-4326, 1973

67. Saddik M, Gamble J, Witters LA, Lopaschuk GD: Acetyl-CoA carboxylase regulation of fatty acid oxidation in the heart. *J Biol Chem* 268:25836-25845, 1993

68. Awan MM, Saggerson DE: Malonyl-CoA metabolism in cardiac myocytes and its relevance to the control of fatty acid oxidation. *Biochem J* 295:61-65, 1993

69. McGarry JD, Mannaerts GP, Foster DW: A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. *J Clin Invest* 60:265-270, 1977

70. Mills SE, Foster DW, McGarry JD: Interaction of malonyl-CoA and related compounds with mitochondria from different rat tissues: relationship between ligand binding and inhibition of carnitine palmitoyltransferase-1. *Biochem J* 214:83-91, 1983

71. Hardie DG: Regulation of fatty acid synthesis via phosphorylation of acetyl-CoA carboxylase. *Prog Lipid Res* 28:117-146, 1989

72. Allred JB, Reilly KE: Short-term regulation of acetyl-CoA carboxylase in tissues of higher animals. *Prog Lipid Res* 35:371-385, 1997

73. Sim AR, Hardie DG: The low activity of acetyl-CoA carboxylase in basal and glucagons-stimulated hepatocytes is due to phosphorylation by the AMP-activated protein kinase and not cyclic AMP-dependent protein kinase. *FEBS Lett* 233:294-298, 1988

74. Kudo N, Gillespie JG, Kung L, Witters LA, Schulz R, Clanachan AS, Lopaschuk GD: Characterization of 5' AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. *Biochim Biophys Acta* 1301:67-75, 1996

75. Winder WW, Wilson HA, Hardie DG, Rasmussen BB, Hutber CA, Call GB, Clayton RD, Conley LM, Yoon S, Zhou B: Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. *J Appl Physiol* 82:219-225, 1997

76. Hardie DG, Carling D: The AMP-activated protein kinase: fuel gauge of the mammalian cell? *Eur J Biochem* 246:259-273, 1997

77. Russell RR 3rd, Bergeron R, Shulman GI, Young LH: Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol* 277:H643-H649, 1999

78. Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L: Phosphorylation and activation of heart

PFK-2 by AMPK has a role in the stimulation of glycolysis during ischemia. *Curr Biol* 10:1247-1255, 2000

79. Muoio DM, Seefeld K, Witters LA, Coleman RA: AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target. *Biochem J* 338:783-791, 1999

80. Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG: Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J Biol Chem* 271:27879-27887, 1996

81. Moore F, Weekes J, Hardie DG: Evidence that AMP triggers phosphorylation as well as direct allosteric activation of rat liver AMP-activated protein kinase. A sensitive mechanism to protect the cell against ATP depletion. *Eur J Biochem* 199:691-697, 1991

82. Ponticos M, Lu QL, Morgan JE, Hardie DG, Partridge TA, Carling D: Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *EMBO J* 17:1688-1699, 1998

83. Davies SP, Hawley SA, Woods A, Carling D, Haystead TAJ, Hardie DG: Purification of the AMP-activated protein kinase on ATP-gamma-sepharose and analysis of its subunit structure. *Eur J Biochem* 223:351-357, 1994

84. Woods A, Cheung PCF, Smith FC, Davison MD, Scott J, Beri RK, Carling D: Characterization of AMP-activated protein kinase beta and gamma subunits. Assembly of the heterotrimeric complex in vitro. *J Biol Chem* 271:10282-10290, 1996

85. Lochhead PA, Salt IP, Walker KS, Hardie DG, Sutherland C: 5-aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase. *Diabetes* 49:896-903, 2000

86. Carling D, Hardie DG: The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *Biochim Biophys Acta* 1012:81-86, 1989

87. Young ME, Radda GK, Leighton B: Activation of glycogen phosphorylase and glycogenolysis in rat skeletal muscle by AICAR—an activator of AMP-activated protein kinase. *FEBS Lett* 382:43-47, 1996

88. Holmes BF, Kurth-Kraczek EJ, Winder WW: Chronic activation of 5'-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. *J Appl Physiol* 87:1990-1995, 1999

89. Aschenbach WG, Hirschman MF, Fujii N, Sakamoto K, Howlett KF, Goodyear LJ. Effect of AICAR treatment on glycogen metabolism in skeletal muscle. *Diabetes* 51:567-573, 2002

90. Wojtaszewski JF, Jorgensen SB, Hellsten Y, Hardie DG, Richter EA: Glycogen-dependent effects of 5-aminoimidazole-4-carboxamide (AICA)-riboside on AMP-activated protein kinase and glycogen synthase activities in rat skeletal muscle. *Diabetes* 51:284-292, 2002

91. Milan D, Jeon JT, Looft C, Amarger V, Robic A, Thelander M, Rogel-Gaillard C, Paul S, Iannuccelli N, Rask L, Ronne H, Lundstrom K, Reinsch N, Gellin J, Kalm E, Roy PL, Chardon P, Andersson L: A mutation in PRKAG3 associated

with excess glycogen content in pig skeletal muscle. *Science* 288:1248-1251, 2000

92. Arad M, Benson DW, Perez-Atayde AR, McKenna WJ, Sparks EA, Kanter RJ, McGarry K, Seidman JG, Seidman CE: Constitutively active AMP kinase mutations cause glycogen storage disease mimicking hypertrophic cardiomyopathy. *J Clin Invest* 109:357-362, 2002

93. Daniel T, Carling D: Functional analysis of mutations in the gamma 2 subunit of AMP-activated protein kinase associated with cardiac hypertrophy and Wolff-Parkinson-White syndrome. *J Biol Chem* Oct 22: [epub ahead of print], 2002

94. Beauloye C, Marsin AS, Bertrand L, Krause U, Hardie DG, Vanoverschelde JL, Hue L: Insulin antagonizes AMP-activated protein kinase activation by ischemia or anoxia in rat hearts, without affecting total adenine nucleotides. *FEBS Lett* 505:348-352, 2001

95. Gamble J, Lopaschuk GD: Insulin inhibition of 5' adenosine monophosphate-activated protein kinase in the heart results in activation of acetyl coenzyme A carboxylase and inhibition of fatty acid oxidation. *Metabolism* 46:1270-1274, 1997

96. Witters LA, Kemp BE: Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase. *J Biol Chem* 267:2864-2867, 1992

97. Salt IP, Johnson G, Ashcroft SJ, Hardie DG: AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release. *Biochem J* 335:533-539, 1998

98. Hickson-Bick DL, Buja ML, McMillin JB: Palmitate-mediated alterations in the fatty acid metabolism of rat neonatal cardiac myocytes. *J Mol Cell Cardiol* 32:511-519, 2000

99. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425-432, 1994

100. Baile CA, Della-Fera MA, Martin RJ: Regulation of metabolism and body fat mass by leptin. *Ann Rev Nutr* 20:105-127, 2000

101. Reidy SP, Weber J: Leptin: an essential regulator of lipid metabolism. *Comp Biochem Physiol A Mol Integr Physiol* 125:285-298, 2000

102. Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F: Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 269:540-543, 1995

103. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM: Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 269:543-546, 1995

104. Tartaglia LA: The leptin receptor. *J Biol Chem* 272:6093-6096, 1997

105. Tartaglia L, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, *et al:* Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83:1263-1271, 1995

106. Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM: Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379:632-635, 1996

107. Wang MY, Zhou YT, Newgard CB, Unger RH: A novel leptin receptor isoform in rat. *FEBS Lett* 392:87-90, 1998

108. Chua SC, Chung WK, Wu-Peng XS: Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science* 271:994-996, 1995

109. Vaisse C, Halaas JL, Horvarth CM, Darnell JE Jr, Stoffel M, Friedman JM: Leptin activation of Stat3 in the hypothalamus of wild type and ob/ob mice but not db/db mice. *Nat Genet* 14:95-97, 1996

110. Ghilardi N, Ziegler S, Wiestner A, Stoffel R, Heim MH, Skoda RC: Defective STAT signaling by the leptin receptor in diabetic mice. *Proc Natl Acad Sci USA* 93:6231-6235, 1996

111. Bjorback C, Uotani S, da Silva B, Flier JS: Divergent signaling capacities of the long and short isoforms of the leptin receptor. *J Biol Chem* 272:32686-32695, 1997

112. Muoio DM, Dohm GL, Fiedorek FT Jr, Tapscott EB, Coleman RA. Leptin directly alters lipid partitioning in skeletal muscle. *Diabetes* 46:1360-1363, 1997

113. Muoio DM, Dohm GL, Tapscott EB, Coleman RA: Leptin opposes insulin's effects on fatty acid partitioning in muscle isolated from obese ob/ob mice. *Am J Physiol* 276:E913-E921, 1999

114. Steinberg GR, Bonen A, Dyck DJ: Fatty acid oxidation and triacylglycerol hydrolysis are enhanced after chronic leptin treatment in rats. *Am J Physiol* 282:E593-E600, 2002

115. Dissociation of obesity and impaired glucose disposal in mice overexpressing acyl coenzyme A:diacylglycerol acyltransferase 1 in white adipose tissue. *Diabetes* 51:3189-3195, 2002

116. Ebihara K, Ogawa Y, Masuzaki H, Shintani M, Miyanaga F, Aizawa-Abe M, Hayashi T, Hosoda K, Inoue G, Yoshimasa Y, Gavrilova O, Reitman ML, Nakao K: Transgenic overexpression of leptin rescues insulin resistance and diabetes in a mouse model of lipoatrophic diabetes. *Diabetes* 50:1440-1448, 2001

117. Colombo C, Cutson JJ, Yamauchi T, Vinson C, Kadowaki T, Gavrilova O, Reitman ML: Transplantation of adipose tissue lacking leptin is unable to reverse the metabolic abnormalities associated with lipoatrophy. *Diabetes* 51:2727-2733, 2002

118. Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, Kahn BB: Leptin stimulates fatty acid oxidation by activating AMP-activated protein kinase. *Nature* 415:339-343, 2002

119. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okumbo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y: Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 257:79-83, 1999

120. Fruebis J, Tsa TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF: Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci USA* 98:2005-2010, 2001

121. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7:941-946, 2001

122. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T: Adiponectin stimulates glucose utilization and fatty acid oxidation by activating AMP-activated protein kinase. *Nat Med* 8:1-8, 2002

123. Ma K, Cabrero A, Saha PK, Kojima H, Li L, Chang BH, Paul A, Chan L: Increased beta-oxidation but no insulin resistance or glucose intolerance in mice lacking adiponectin. *J Biol Chem* 277:34658-34661, 2002

124. Neely JR, Morgan HE: Relationship between carbohydrate and lipid metabolism and the energy balance of the heart muscle. *Ann Rev Physiol* 36:415-439, 1974

125. Lopaschuk GD: Metabolic abnormalities in the diabetic heart. *Heart Fail Rev* 7:149-159, 2002

126. Belke DD, Larsen TS, Gibbs EM, Severson DL: Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (db/db) mice. *Am J Physiol* 279:E1104-E1113, 2000

127. Echocardiographic assessment of cardiac function in diabetic db/db and transgenic db/db-hGLUT4 mice. *Am J Physiol* 283:H976-H982, 2002

128. Young ME, Guthrie PH, Razeghi P, Leighton B, Abbasi S, Patil S, Youker KA, Taegtmeyer H: Impaired long-chain fatty acid oxidation and contractile dysfunction in the obese Zucker rat heart. *Diabetes* 51:2587-2595, 2002

129. Zhou YT, Grayburn P, Karim A, Shimabukuro M, Higa M, Baetens D, Orci L, Unger RH: Lipotoxic heart disease in obese rats: implications for human obesity. *Proc Natl Acad Sci USA* 97:1784-1789, 2000

130. Chiu HC, Kovacs A, Ford DA, Hsu F-F, Garcia R, Herrero P, Saffitz JE, Schaffer JE: A novel mouse model of lipotoxic cardiomyopathy. *J Clin Invest* 107:813-822, 2001

131. Corr PB, Creer MH, Yamada KA, Saffitz JE, Sobel BE: Prophylaxis of early ventricular fibrillation by inhibition of acylcarnitine accumulation. *J Clin Invest* 83:927-936, 1989

132. Regan TJ, Weisse AB: Diabetic cardiomyopathy. *J Am Coll Cardiol* 19:1165-1166, 1992

133. Zoneraich S, Mollura JL. Diabetes and the heart: state of the art in the 1990's. *Can J Cardiol* 9:293-299, 1993

134. York DA: Lessons from animal models of obesity. *Endocrinol Metab Clin North Am* 25:781-800, 1996

135. Chalkley SM, Hettiarachchi M, Chisholm DJ, Kraegan EW: Long-term high-fat feeding leads to severe insulin resistance but not diabetes in Wistar rats. *Am J Physiol* 282:E1231-E1238, 2002

136. daSilva BA, Bjorbaek C, Uotani S, Flier JS: Functional properties of leptin receptor isoforms containing the gln→pro extracellular domain mutation of the fatty rat. *Endocrinology* 139:3681-3690, 1998

137. Russell JC, Shillabeer G, Bar-Tana J, Lau DCW, Richardson M, Wenzel LM, Graham SE, Dolphin PJ: Development of insulin resistance in the JCR:LA-cp rat: Role of triacylglycerols and effects of MEDICA 16. *Diabetes* 47:770-778, 1998

138. Wu-Peng XS, Chua SC Jr, Okada N, Liu SM, Nicolson M, Leibel RL: Phenotype of the obese Koletsky (f) rat due to Tyr763Stop mutation in the extracellular domain of the leptin receptor (Lepr). *Diabetes* 46:513-518, 1997

139. Russell JC, Koeslag DG, Dolphin PJ, Amy RM: Prevention of myocardial lesions in JCR:LA-cp corpulent rats by nifedipine. *Arteriosclerosis* 10:658-664, 1990

140. Rose-Kahn G, Bar-Tana J: Inhibition of rat liver acetyl-CoA carboxylase by beta, beta'-tetramethyl-substituted hexadecanedioic acid (MEDICA 16). *Biochim Biophys Acta* 1042:259-264, 1990

141. Mayorek N, Kalderon B, Itach E, Bar-Tana J: Sensitization to insulin induced by beta, beta'-methyl-substituted hexadecanedioic acid (MEDICA 16) in obese Zucker rats in vivo. *Diabetes* 46:1958-1964, 1997

142. Frenkel B, Bishara-Shieban J, Bar-Tana J: The effect of β,β'-tetramethylhexadecanedioic acid (MEDICA 16) on plasma very-low-density lipoprotein metabolism in rats: role of apolipoprotein C-III. *Biochem J* 298:409-414, 1994

143. Bar-Tana J, Rose-Kahn G, Srebnik M: Inhibition of lipid synthesis by $\beta\beta$'-tetrametyl-substituted, C14-C22, α,ω-dicarboxylic acids in the rat in vivo. *J Biol Chem* 260:8404-8410, 1985

144. Abu-Elheiga L, Matzuk MM, Abo-Hashema KA, Wakil SJ: Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. *Science* 291:2613-2616, 2001

145. Moller DE: New drug targets for type 2 diabetes and the metabolic syndrome. *Nature* 414:821-827, 2001

146. Winder WW: AMP-activated protein kinase: possible target for treatment of type 2 diabetes. *Diabetes Technol Ther* 2:441-448, 2000

147. Winder WW, Hardie DG: AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* 277:E1-E10, 1999

148. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE: Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108:1167-1174, 2001

149. Fryer LG, Parbu-Patel A, Carling D: The anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. *J Biol Chem* 277:25226-25232, 2002

150. Hawley SA, Gadalla AE, Olsen GS, Hardie DG: The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* 51:2420-2425, 2002

151. Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, Zhou G, Williamson JM, Ljunqvist O, Efendic S, Moller DE, Thorell A,

Goodyear LJ: Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes* 51:2074-2081, 2002

152. Iglesias MA, Ye JM, Frangioudakis G, Saha AK, Tomas E, Ruderman NB, Cooney GJ, Kraegen EW: AICAR administration causes an apparent enhancement of muscle and liver action in insulin-resistant high-fat fed rats. *Diabetes* 51:2886-2894, 2002

153. Bergeron R, Previs SF, Cline GW, Perret P, Russell RR 3rd, Young LH, Shulman GI: Effect of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside infusion on in vivo glucose and lipid metabolism in lean and obese Zucker rats. *Diabetes* 50:1076-1082, 2001

154. Fiedler M, Zierath JR, Selen G, Wallberg-Henriksson H, Liang Y, Sakariassen KS: 5-aminoimidazole-4-carboxy-amide-1-beta-D-ribofuranoside treatment ameliorates hyperglycaemia and hyperinsulinemia but not dyslipidemia in KKAy-CETP mice. *Diabetologia* 44:2180-2186, 2001

155. Song XM, Fiedler M, Galuska D, Ryder JW, Fernstrom M, Chibalin AV, Wallberg-Henriksson H, Zierath JR: 5-Aminoimidazole-4-carboxamide ribonucleoside treatment improves glucose homeostasis in insulin resistant diabetic (ob/ob) mice. *Diabetologia* 45:56-65, 2002

156. Halseth AE, Ensor NJ, White TA, Ross SA, Gulve EA: Acute and chronic treatment of ob/ob and db/db mice with AICAR decreases blood glucose concentrations. *Biochem Biophys Res Commun* 294:798-805, 2002

157. Buhl ES, Jessen N, Pold R, Ledet T, Flyvbjerg A, Pedersen SB, Pedersen Q, Schmitz O, Lund S: Long-term AICAR administration reduces metabolic disturbances and lowers blood pressure in rats displaying features of insulin resistance syndrome. *Diabetes* 51:2199-2206, 2002

158. Gollob MH, Seger JJ, Gollob TN, Tapscott T, Gonzales O, Bachinski L, Roberts R: Novel PRKAG2 mutation responsible for the genetic syndrome of ventricular preexcitation and conduction system disease with childhood onset and absence of cardiac hypertrophy. *Circulation* 104:3030-3033, 2001

159. Gollob MH, Green MS, Tang AS, Gollob T, Karibe A, Ali Hassan AS, Ahmad F, Lozado R, Shah G, Fananapazir L, Bachinski LL, Roberts R, Hassan AS: Identification of a gene responsible for familial Wolff-Parkinson-White syndrome. *N Engl J Med* 344:1823-1831, 2001

160. Blair E, Redwood C, Ashrafian H, Oliveira M, Broxholme J, Kerr B, Salmon A, Ostman-Smith I, Watkins H: Mutations in the gamma(2) subunit of AMP-activated protein kinase cause familial hypertrophic cardiomyopathy: evidence for the central role of energy compromise in disease pathogenesis. *Hum Mol Genet* 10:1215-1220, 2001

161. Tian R, Musi N, D'Agostino J, Hirshman MF, Goodyear LJ: Increased adenosine monophosphate-activated protein kinase activity in rat hearts with pressure-overload hypertrophy. *Circulation* 104:1664-1669, 2001

162. Hamilton SR, Stapleton D, O'Connell JB Jr, Kung JT, Dalal SR, Kemp BE, Witters LA: An activating mutation in the gamma subunit of the AMP-activated protein kinase. *FEBS Lett* 500:163-168, 2001

163. Henriksen EJ: Invited Review: Effects of acute exercise and exercise training on insulin resistance. *J Appl Physiol* 93:788-796, 2002

164. Pessin JE, Saltiel AR: Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest* 106:165-169, 2000

165. Tomas E, Zorzano A, Ruderman NB: Invited Review: Exercise and insulin signaling: a historical perspective. *J Appl Physiol* 93:765-772, 2002

166. Zierath JR: Invited Review: Exercise training-induced changes in insulin signaling in skeletal muscle. *J Appl Physiol* 93:773-781, 2002

167. Stephens TJ, Chen ZP, Canny BJ, Michell BJ, Kemp BE, McConnell GK: Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise. *Am J Physiol* 282:E688-E694, 2002

168. Musi N, Fujii N, Hirschman MF, Ekberg I, Froberg S, Ljungqvist O, Thorell A. Goodyear LJ: AMP-activated protein kinase (AMPK) is activated in muscle of subjects with type 2 diabetes during exercise. *Diabetes* 50:921-927, 2001

169. Abel EL: Physical activity does not account for the physiological response to forced swim testing. *Physiol Behav* 56:677-681, 1994

170. Russell JC, Epling WF, Pierce D, Amy RM, Boer DP: Induction of voluntary prolonged running by rats. *J Appl Physiol* 63:2549-2553, 1987

CHAPTER 2. MATERIALS AND METHODS

CHAPTER 2. MATERIALS AND METHODS

Materials

[1-^{14}C]glucose, D-[5-^{3}H]glucose was obtained from Amersham Canada Ltd (Oakville, Ontario). [9,10-^{3}H]palmitic acid and [1-^{14}C]palmitic acid were obtained from NEN (Boston, MA). [^{3}H]sn-glycerol-3-phosphate was obtained from NEN (Boston, MA). L-[methyl-^{3}H]carnitine HCl was obtained from Amersham Biosciences Corporation (Quebec, Canada). Bovine serum albumin (BSA fraction V, fatty acid free) was obtained from Boehringer Mannheim (Indianapolis, IN). Hyamine hydroxide (1M in methanol solution) was obtained from NEN Research Products (Boston, MA). ACS Aqueous Counting Scintillant was obtained from Amersham Canada Ltd (Oakville, Ontario). MEDICA 16 was a gift from Dr. Jacob Bar-Tana (Jerusalem, Israel). Triglyceride and Free Fatty Acid assay kits were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Leptin immunoassay kit was purchased from R&D Systems (Minneapolis, MN). For HPLC analysis of malonyl-CoA, a Novo-Pak 4 μm, 60A°, C18 precolumn cartridge was purchased from Waters Company (Milford, MA) and an Absorbosphere C18, 3 μm, 150 mm X 4.6 mm column was purchased from Alltech Associates/Mandel Scientific (Guelph, ON). ECL Western blotting detection reagents were purchased from Amersham International (Amersham, UK). Monoclonal or polyclonal antibodies to cytosolic H-FABP and phospho-AMPK were purchased from Signal Transduction Laboratories. Secondary antibodies (peroxidase conjugated affinipore goat anti-mouse IgG and peroxidase conjugated affinipore goat anti-rabbit IgG) were purchased from Jackson Immunoresearch Laboratories Inc. (Mississauga, Ontario). Peroxidase labeled streptavidin was purchased through Mandel Scientific from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). Trans-Blot Transfer Medium (PVDF membrane) was obtained from BioRad (Richmond, CA). X-ray films (X-OMAT AR Film) were purchased from Kodak (Rochester, NY). All other chemicals were obtained from the Sigma Chemical Company (St. Louis, MO).

Methods

Sprague Dawley Rats

Adult male Sprague Dawley rats weighing between 250-300g were obtained from the University of Alberta.

JCR:LA-cp Rats

4 and 12 week old JCR:LA-cp (cp/cp) and lean age-matched controls (either +/cp or +/+) were bred in an established colony at the University of Alberta. Animals were maintained in a controlled environment at 20^0 C and 50-55% relative

humidity, with a 12:12 h light-dark cycle. Animals were maintained on rat chow (Rodent Diet 5001; PMI Nutrition International) and distilled water was available *ad libitum*. Animals were either fasted or fed prior to sacrifice as described in individual chapters. Care and treatment of the rats conformed to the guidelines of the Canadian Council on Animal Care and was subject to prior institutional approval as provided for in the guidelines.

JCR:LA-cp Rats (Exercise Study)

4-week old JCR:LA-cp (cp/cp) were bred in an established colony at the University of Alberta. Animals were maintained in a controlled environment at 20^0C and 50-55% relative humidity, with a 12:12 h light-dark cycle. Animals were maintained on rat chow (Rodent Diet 5001; PMI Nutrition International) and distilled water as described in Chapter 6.

MEDICA 16 Treatment

In one series of experiments described in Chapter 5, JCR:LA-cp rats were treated for 6 weeks with MEDICA 16. Body weight and food consumption were measured twice weekly. MEDICA 16 was incorporated in the rat chow at a concentration of 0.25% (wt/wt).

Blood Collection

Blood samples were either obtained by cardiac puncture or by sampling from the tail vein as described in Chapters 4, 5 and 6. Samples were cooled on ice for approximately 15 minutes and then centrifuged at 1,500 X g for 15 minutes. Following centrifugation, the top layer (plasma) was removed at stored at -70^{O}C for further analysis.

Plasma Measurements

Samples were analyzed for plasma glucose using a glucose oxidase technique (Beckman Instruments, Brea, CA). Insulin levels were determined by radioimmunoassay (Insulin RIA 100, Kabi Pharmacia, Uppsala, Sweden, with rat insulin standards). Plasma free fatty acid levels were measured using an enzymatic colorimetric assay (Wako® Pure Chemical Industries, Osaka, Japan). Plasma TG levels were measured using an enzymatic colorimetric assay (Wako® Pure Chemical Industries, Osaka, Japan). Plasma leptin levels were determined by immunoassay (R&D Systems, Minneapolis, MN).

Meal Tolerance Test

A standardized meal tolerance test (MTT) provides a sensitive index of insulin and glucose metabolism in the insulin resistant JCR:LA-cp rats (1). After an overnight fast, rats were warmed on a heated table to ensure vasodilation of the tail, and a 0.25 - 0.5 ml blood sample (depending on the weight of the animal) was obtained from the tip of the tail. The rats were then replaced in their cage and given 5 g of food. When 50% of the food was eaten, timing was started and subsequent blood samples were taken at 30 and 60 minutes.

Exercise Protocol

The study was done on JCR:LA-cp (cp/cp) male rats beginning at the age of 4-weeks. During the 4-week experimental protocol (described in detail in Chapter 6), body weight, food intake and water intake were recorded daily. Fasting blood samples were obtained from the tail vein prior to the start of the experiment, at 6.5 weeks and at 8.5 weeks of age. A meal tolerance test to determine insulin sensitivity was performed at 8.5 weeks of age.

During the 8 week experimental protocol (described in detail in Chapter 6), all groups were subjected to the 4-week experimental protocol as described above. However, at 8.5 weeks of age, exercise-training and weight-matching were stopped and all groups received free access to food and water for the remaining 4-weeks. Body weight, food intake and water intake were recorded daily. Fasting blood samples were obtained from the tail vein prior to the start of the experiment, at 6.5, 8.5, 10.5 and 12.5 weeks of age. A meal tolerance test to determine insulin sensitivity was performed at 12.5 weeks of age.

Wheel Revolutions

Exercise-trained and weight-paired JCR:LA-cp rats were housed in Wahman running wheels (1.1 meter circumference) as described previously (2). However, the wheels, in cages of the weight-paired group, were locked. Animals were put in the running wheels 4 days prior to the beginning of the experimental protocol. The wheels were continuously monitored through a computer and the daily number of complete wheel revolutions recorded.

Heart Perfusions

For the leptin study described in Chapter 3, adult male Sprague Dawley rats (250-300 g) were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). For all other studies, adult male JCR:LA-cp and lean controls were anesthetized without restraint in a large glass jar with 3% halothane at 1 l/min O_2. Hearts were rapidly excised and placed in ice-cold Krebs-Henseleit solution. The aorta was quickly cannulated and perfusion was initiated using Krebs-Henseleit solution containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$ and 2.5 mM $CaCl_2 \cdot 2H_2O$. At this time, excess tissue was removed from the heart and the opening to the left atrium was cannulated. After a 10 minute equilibration period, hearts were switched to the working heart mode. Left atrial preload was 11.5 mmHg and aortic afterload was 80 mmHg. Spontaneously beating hearts were used throughout the studies. Heart rate and peak systolic pressure (PSP) were measured using a Gould P21 pressure transducer in the aortic outflow line. Cardiac output, aortic flow and coronary flow were measured using Transonic in-line ultrasonic flow probes connected to T101 ultrasonic blood flow meter. Mechanical function was monitored throughout the entire perfusion period. Cardiac work is expressed as the product of peak systolic pressure X cardiac output.

Perfusion Protocols

For the leptin study described in Chapter 3, isolated working hearts from male Sprague Dawley rats were subjected to aerobic perfusion with a modified Krebs-Henseleit solution containing 0.8 mM palmitate, 5 mM glucose, 3% bovine serum albumin, 2.5 mM free Ca^{2+}, in the absence or presence of 100 μU/ml (600 pmol/l) insulin. The hearts, in which fatty acid oxidation and glucose oxidation rates were measured, were subjected to a 60-minute perfusion with perfusate containing [9,10-^{3}H]palmitate and [1-^{14}C]glucose. Palmitate and glucose oxidation rates were determined simultaneously by the quantitative collection of ^3H_2O and $^{14}CO_2$ produced by the heart, as described previously (3). Steady-state fatty acid and glucose oxidation rates were calculated from the linear time course as the mean of nmol [^{3}H]palmitate $\cdot$ g dry wt $^{-1}$ $\cdot$ min $^{-1}$ or nmol [^{14}C]glucose $\cdot$ g dry wt $^{-1}$ $\cdot$ min $^{-1}$, respectively, for each 10 minute sampling time point during the 60-minute perfusion. Acetyl-CoA production was calculated as the total amount of palmitate or glucose oxidized $\cdot$ gram dry wt $^{-1}$ * acetyl-CoA produced with palmitate producing 8 acetyl-CoA/palmitate and glucose producing 2 acetyl-CoA/glucose.

For studies described in Chapters 4 and 6, isolated working hearts from JCR:LA-cp and lean control rats were subjected to aerobic perfusion with a modified Krebs-Henseleit solution containing 0.8 mM palmitate, 5 mM glucose, 500 μU/ml (3000 pmol/l) insulin, 3% bovine serum albumin, and 2.5 mM free Ca^{2+}. The hearts, in which fatty acid oxidation and glycolytic rates were measured, were subjected to a 40-minute perfusion with perfusate containing [1-^{14}C]palmitate and D-[5-^{3}H]glucose. Palmitate oxidation and glycolytic rates were determined simultaneously by the quantitative collection of $^{14}CO_2$ and $^{3}H_2O$ produced by the hearts, as described previously (3). Steady-state fatty acid oxidation and glycolytic rates were calculated from the linear time course as the mean of the nmol [^{14}C]palmitate · g dry wt $^{-1}$ · min $^{-1}$ or μmol [^{3}H]glucose · g dry wt^{-1} · min $^{-1}$, respectively, for each 10 minute sampling time point during the 40-minute perfusion.

Tissue Analysis

At the end of the perfusions, heart ventricles were rapidly frozen with Wollenberger clamps cooled to the temperature of liquid N_2. The frozen ventricular tissue was then weighed and powdered in a mortar and pestle cooled to the temperature of liquid N_2. A portion of the powdered tissue was used to determine the dry wt to wet wt ratio of the ventricles. The atrial tissue remaining on the cannula was removed, dried in an oven for 12 hr at 100^{O}C, and weighed. The dried atrial weight, frozen ventricular weight, and ventricular dry to wet ratio were then used to determine the total dry weight of the heart.

Tissue Collection

JCR:LA-cp rats were handled twice a week from birth to ensure minimal stress at the time of sacrifice. Rats were anesthetized without restraint in a large glass jar with 3% halothane at 1 l/min O_2. This procedure allowed minimal stress to the animal prior to tissue sampling. The time to reach surgical plane with halothane varied slightly, with heavier animals taking slightly longer. Tissues (muscle, heart, liver) were harvested within 30 seconds after surgical plane was reached, frozen immediately in liquid N_2, and stored at -80^{O}C until analysis.

Tissue Triacylglycerol Determination

For studies described in Chapter 4 and 5, TG was extracted from frozen tissue according to the method of Folch (4). Briefly, 20 mg of frozen heart tissue was extracted in a 20-fold volume of 2:1 chloroform:methanol following which a 0.2 volume of methanol was added, and the extract vortexed for 30 seconds. The mixture was then centrifuged at 1,100 X g for 10 minutes and the supernatant collected. The resulting supernatant was applied to a silicic acid column. TG was eluted in 10 volumes of chloroform, dried under N_2 at 60^{O}C, and quantitated colorimetrically using an enzymatic assay (Wako® Pure Chemical Industries).

For studies described in Chapters 3 and 6, TG was extracted from frozen tissue according to the method of Folch (4). Briefly, 20 mg of frozen heart tissue was extracted in a 20-fold volume of 2:1 chloroform:methanol following which a 0.2 volume of methanol was added, and the extract vortexed for 30 seconds. The mixture was then centrifuged at 1,100 X g for 10 minutes and the supernatant collected. A 0.2 volume of 0.04% $CaCl_2$ was added to the supernatant which was then centrifuged at 550 X g for 20 minutes. The upper phase was then removed and the interface was washed 3 times with pure solvent upper phase consisting of 1.5 ml chloroform, 24.0 ml methanol, and 23.5 ml water. The final wash was removed and 50 μl of methanol was added to obtain one phase. The samples were then dried under N_2 at 60^0C and redissolved in 50 μl of 3:2 tert-butyl alcohol:triton X-100 (5). Cardiac TG was then quantitated colorimetrically using an enzymatic assay (Wako® Pure Chemical Industries).

[^{3}H] Palmitate Incorporation into Triacylglycerol

In the studies described in Chapter 3, palmitate incorporation into TG was determined using an aliquot from the total TG isolated as described above. Total cardiac TG was resuspended in 4 ml scintillation fluid and then counted for the presence of radiolabelled [^{3}H]palmitate. Total (exogenous and endogenous) fatty acid oxidation was determined by the following equation: [total [^{3}H]palmitate label incorporation into TG during the perfusion] + [the change in fatty acid content in TG during the perfusion] + [total [^{3}H]palmitate oxidized during the perfusion].

This calculation makes the following assumptions: 1) TG pool size was similar in control and leptin-treated hearts at the onset of leptin administration, 2) the TG pool size remains constant in the control hearts throughout the perfusion period, which we have shown to occur under these perfusion conditions (6), 3) the amount of [^{3}H]palmitate label entering the TG pool equals that of endogenous or unlabelled fatty acid leaving the pool and 4) all palmitate released from the TG pool is oxidized. Previous studies of TG turnover and the contribution of TG to energy substrate utilization in isolated working rat hearts demonstrate that these assumptions are valid (6,7).

CoA Ester Levels

Approximately 20 mg of frozen heart tissue was extracted in 200 μl 6% perchloric acid containing 1 mM dithiothreitol (to prevent oxidation during isolation). Following homogenization, samples were left on ice for 10 minutes and then centrifuged at 13,400 X g for 5 minutes. The supernatant was removed and subjected to HPLC analysis as described previously (8).

Polyethylene Glycol (PEG) Precipitation

A cytosolic fraction containing ACC and AMPK was isolated from frozen tissue according to the polyethylene glycol (PEG) precipitation method (9). 200 mg of frozen tissue was homogenized in homogenization solution containing 50 mM Tris-HCl (pH 7.5 at 4^0C), 50 mM NaF, 5 mM Na^+ pyrophosphate, 1 mM

EDTA, 1 mM EGTA, 0.25 M mannitol, 1 mM DTT and a protease inhibitor cocktail (Sigma®). After homogenization for 30 seconds, the mixture was centrifuged at 14,000 X g for 20 minutes. The resulting supernatant was made to 2.5% PEG, vortexed for 10 minutes and centrifuged for 10 minutes at 10,000 X g. The resulting supernatant was then made to 6% PEG, vortexed for 10 minutes and centrifuged for 10 minutes at 10,000 X g. The resulting pellet was resuspended in solution containing 0.1 M Tris-HCl (pH 7.5 at 4^0C), 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 10% w/v glycerol, 1 mM DTT, 0.02% sodium azide and the protease inhibitor cocktail (Sigma® P8340). The protease inhibitor cocktail is a mixture of protease inhibitors with broad specificity for the inhibition of serine, cysteine, aspartic proteases and aminopeptidases. Protein content was measured using the Bradford protein assay (10)

Whole Cell Homogenate Preparation

Whole cell homogenates were obtained by homogenizing 50 mg of frozen heart tissue in homogenization solution containing 0.1 M Tris-HCl (pH 7.5 at 4 ^{0}C), 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 10% w/v glycerol, 1 mM DTT, 0.02% sodium azide and the protease inhibitor cocktail (Sigma®). After homogenization for 30 seconds, the mixture was centrifuged at 800 X g for 10 minutes. Protein content was measured in the resulting supernatant using the Bradford protein assay (10).

ACC Activity

The "CO_2 fixation technique" was used to measure ACC activity in the 6% PEG fraction (9). Briefly, 12.5 ug of protein was added to a reaction mixture (final volume 190 μl) containing 60.6 mM Tris acetate, 1 mg/ml BSA, 1.32 μM ATP, 2.12 mM β-mercaptoethanol, 5 mM magnesium acetate, 1.06 mM acetyl-CoA, 18.08 mM $NaH^{14}CO_3$ and 0 or 10 mM magnesium citrate. Samples were incubated at 37^0C for 4 minutes and the reaction was stopped by adding 25 μl of 10% perchloric acid. Samples were spun at 1,100 X g for 20 minutes and then 160 μl of supernatant was placed in glass vials and dried under warm heat. The samples were resuspended in 100 μl of ddH_2O and 4 ml scintillation fluid and then counted for the presence of radiolabelled malonyl-CoA. ACC activity is expressed as nmol malonyl-CoA produced $min^{-1} \cdot mg\ protein^{-1}$.

AMPK Activity

AMPK activity of the 6% PEG precipitate was measured by following the incorporation of ^{32}P into the synthetic peptide HMRSAMSGLHLVKRR (SAMS) or AMARAASAAALARRR (AMARA) as described in the individual Chapters (9). Both synthetic peptides are specific substrates for AMPK, however, SAMS detects α1-AMPK activity to a greater extent than α2-AMPK activity. AMARA detects both α1- and α2-AMPK activity equally (11). SAMS was used in Chapter 5 because liver contains predominately the α1-catalytic isoform of AMPK. AMARA was used in chapter 3, 4 and 6 because the α2-catalytic isoform of

AMPK is more predominant in heart. The assay mixture contained 40 mM HEPES pH 7.0, 80 mM NaCl, 0.8 mM EDTA, 1 mM DTT, 0.2 mM [γ-^{32}P]ATP, 5.0 mM $MgCl_2$, 0.2 mM SAMS or AMARA, 8% glycerol, 0.01% Triton X-100, and 2 µg of the PEG precipitate in the presence or absence of 200 µM AMP. Incorporation of ^{32}P into the SAMS or AMARA peptide was measured at 30^{0}C for 5 minutes. An aliquot of the reaction mixture was then blotted onto phosphocellulose paper, washed in H_3PO_4 four times and once in acetone. The phosphocellulose was dried and counted in 4 ml of scintillant. AMPK activity is expressed as pmol ^{32}P incorporated min $^{-1}$ · mg protein $^{-1}$.

GPAT Activity

GPAT activity in whole cell homogenates was assayed by following the incorporation of [^{3}H]sn-glycerol-3-phosphate into lysophosphatidate as described by Swanton *et al.* (12). All assays were performed for 15 minutes in a final volume of 200 µl. Reactions were performed at 30°C in a reaction mixture containing 75 mM Tris-HCl (pH 7.4), fatty acid free bovine serum albumin (1.75 mg/ml), 1 mM dithiothreitol, 1 mM [^{3}H]sn-glycerol-3-phosphate, 40 µM palmitoyl-CoA and 150 µg of homogenate protein. The assay was performed both in the absence or presence of 10 mM N-ethylmaleimide (NEM) to inhibit the microsomal isoform. GPAT activity is expressed as pmol [^{3}H]sn-glycerol-3-phosphate · min $^{-1}$ · mg protein $^{-1}$.

Preparation of Mitochondria

Freshly isolated hearts from JCR:LA-cp and lean control rats were excised and finely minced with scissors in 10 ml of homogenization solution containing 0.25 M sucrose, 5 mM Tris-HCl and 1 mM EGTA, pH 7.4 at 4°C. The crude homogenate was centrifuged at 800 X g for 10 minutes at 4°C. The resulting pellet was washed in 2 volume of homogenization solution and was then re-centrifuged at 800 X g for 10 minutes; this step was repeated twice to maximize the yield of mitochondria. The combined supernatants were centrifuged at 6,000 X g for 15 minutes. The pellet was carefully resuspended in 2 ml of homogenization solution, and centrifuged at 6,000 X g for 15 minutes. The resultant pellet (crude mitochondria) was gently resuspended in 2 ml of homogenization solution. The crude mitochondrial fraction (0.5 ml) was layered onto 9 ml of 30% Percoll and centrifuged at 50,000 X g for 60 minutes at 4 °C. The bottom mitochondrial protein band was collected and used for CPT-I activity determinations.

CPT I Activity

CPT I activity was assayed in the direction of acylcarnitine formation using palmitoyl-CoA and carnitine as substrates (13). Reactions were carried out at 30°C in glass culture tubes (13mm x 100mm). Final concentrations in a total of 500 μl were: 75 μM palmitoyl-CoA, 4 mM ATP, 4 mM $MgCl_2$, 0.25 mM glutathione, 40 μg/ml rotenone, 2 mM KCN, 15 mM KCl, 1 % (w/v) bovine serum albumin and 105 mM Tris-HCl, pH 7.4. The amount of the mitochondrial protein ranged between 35 and 40 μg in a total volume of 50 μl. Palmitoyl-CoA was dissolved in 25 mM KH_2PO_4, pH 5.3. The mitochondria were initially incubated with the assay solution and palmitoyl-CoA for 3 minutes at 30°C. Reactions were initiated by the addition of L-[methyl-^{3}H]carnitine HCl to a final L-carnitine concentration of 200 μM and the incubation continued for a further 6 minutes. Reactions were stopped by adding 100 μl of concentrated HCl. The [^{3}H]palmitoylcarnitine formed was extracted using 1-butanol (14). The incubation mixture (500 μl + 100 μl HCl) was diluted to 2 ml with butanol-saturated water, and [^{3}H]palmitoylcarnitine was extracted with 1 ml of water-saturated butanol. The mixture was vortexed and centrifuged at 1,100 X g for 7 minutes. Butanol phase was removed and washed twice with 2x of its volume with butanol-saturated water. Finally, the radioactivity in 500 μl of the butanol extract was measured using standard scintillation techniques, and the content of palmitoylcarnitine was calculated from the known specific activity of carnitine used.

Immunoblotting

Whole cell homogenates (for cytosolic H-FABP and P-AMPK) or 6% PEG precipitants (for ACC) were diluted in protein sample buffer containing 10% glycerol, 1% β-mercaptoethanol, 2% SDS, 0.1 mg/ml bromophenol blue, 43 mM Tris (pH 6.8), boiled for 5 minutes and then subjected to polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). Samples were resolved by gel electrophoresis using a Mini Trans-Blot Cell (BioRad®) in protein reservoir buffer containing 25 mM Tris (pH 8.3), 0.192 M glycine, and 0.1% sodium dodecylsulfate. Proteins were then transferred to a PVDF (polyvinylidene difluoride) membrane using Towbin's transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol (15). Membranes were blocked at room temperature for 1 hour in either 5% bovine serum albumin or milk powder in Tris-buffered saline containing 0.1% Tween. Membranes were then incubated at 4°C overnight in the primary antibody diluted in blocking buffer. The membranes were then incubated in the appropriate secondary antibody conjugated to horseradish peroxidase at room temperature for 1 hour. Protein expression was visualized using the ECL® Western blot detection kit.

Densitometric Analysis

X-ray films were scanned using a GS-800 Calibrated Densitometer and analyzed using Quantity One® software. As a control, one sample was loaded on all gels and the relative density of the bands determined with Quantity One software was standardized to the control sample. This eliminated differences in the transfer of proteins to the membrane as well as any differences in the level of exposure on the x-ray film. The density of the bands is expressed as relative density standardized to the control sample.

Statistical Analysis

All data are presented as the mean ± standard error (n). The data were analyzed with the statistical program Instat 2.01. Statistical tests are described in detail in the individual chapters. Two-tailed values of $p<0.05$ were considered significant.

References

1. Russell JC, Graham SE, Dolphin PJ: Glucose tolerance and insulin resistance in the JCR:LA-corpulent rat: Effect of miglitol (Bay m1099). *Metabolism* 48:701-706, 1999

2. Morse AD, Russell JC, Hunt TW, Wood GO, Epling WF, Pierce WD: Diurnal variation of intensive running in food-deprived rats. *Can J Physiol Pharmacol* 73:1519-1523, 1995

3. Lopaschuk GD, and Barr RL: Measurements of fatty acid and carbohydrate metabolism in the isolated working rat heart. *Mol Cell Biochem* 172:137-147, 1997

4. Folch J, Lees M, Stanley GH: A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:497-509, 1957

5. Danno H, Jincho Y, Budiyanto S, Furukawa Y, Kimura S: A simple enzymatic quantitative analysis of triglycerides in tissues. *J Nutr Sci Vitaminol* 38:517-521,1992

6. Saddik M, and Lopaschuk GD: Myocardial triglyceride turnover and contribution to energy substrate utilization in isolated working rat hearts. *J Biol Chem* 266:8162-8170, 1991

7. Saddik M, and Lopaschuk GD: Myocardial triglyceride turnover during reperfusion of isolated rat hearts subjected to a transient period of global ischemia. *J Biol Chem* 267:3825-3831, 1992

8. King MT, Reiss PD: Separation and measurement of short-chain coenzyme-A compounds in rat liver by reversed-phase high-performance liquid chromatography. *Anal Biochem* 146:173-179, 1985

9. Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD: High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *J Biol Chem* 270:17513-17520, 1995

10. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254, 1976

11. Woods A, Salt I, Scott J, Hardie DG, Carling D: The α1 and α2 isoforms of the AMP-activated protein kinase have similar activities in rat liver but exhibit differences in substrate specificity in vitro. *FEBS Lett* 397:347-351, 1996

12. Swanton EM, and Saggerson ED: Glycerolipid metabolizing enzymes in rat ventricle and in cardiac myocytes. *Biochim Biophys Acta* 1346:93-102, 1997

13. McGarry JD, Brown NF: Reconstitution of purified, active and malonyl-CoA sensitive rat liver carnitine palmitoyltransferase 1: relationship between membrane environment and malonyl-CoA sensitivity. *Biochem J* 349:179-187, 2000

14. Bremer J, Norum KR: The mechanism of substrate inhibition of palmityl coenzyme A:carnitine palmitoyltransferase by palmityl coenzyme A. *J Biol Chem* 242:1744-1748, 1967

15. Towbin J, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350-4354, 1979

CHAPTER 3.

Leptin Activates Cardiac Fatty Acid Oxidation Independent of Changes in the AMP-Activated Protein Kinase-Acetyl-CoA Carboxylase-Malonyl-CoA Axis

---

A version of this chapter has been published. Atkinson LL, Fischer MA, Lopaschuk GD. *J Biol Chem* 277:29424-29430, 2002

My role in this work involved performing all the biochemical experiments (except those noted below) as well as writing the manuscript. Melanie A. Fischer performed the heart perfusions. Ken Strynadka performed the HPLC analysis of CoA esters.

CHAPTER 3.

Leptin Activates Cardiac Fatty Acid Oxidation Independent of Changes in the AMP-Activated Protein Kinase-Acetyl-CoA Carboxylase-Malonyl-CoA Axis

Abstract

Leptin regulates fatty acid metabolism in liver, skeletal muscle and pancreas by partitioning fatty acids into oxidation rather than triacylglycerol (TG) storage. Although leptin receptors are present in the heart, it is not known whether leptin also regulates cardiac fatty acid metabolism. To determine whether leptin directly regulates cardiac fatty acid metabolism, isolated working rat hearts were perfused with 0.8 mM [9,10-^{3}H]palmitate and 5 mM [1-^{14}C]glucose to measure palmitate and glucose oxidation rates. Leptin (60 ng/ml) significantly increased palmitate oxidation rates 60% above control hearts ($p<0.05$) and decreased TG content by 33% ($p<0.05$) over the 60-minute perfusion period. In contrast, there was no difference in glucose oxidation rates between leptin-treated and control hearts. Although leptin did not affect cardiac work, oxygen consumption increased by 30% ($p<0.05$) and cardiac efficiency was decreased by 42% ($p<0.05$). AMP-activated protein kinase (AMPK) plays a major role in the regulation of cardiac

fatty acid oxidation by inhibiting acetyl-CoA carboxylase (ACC) and reducing malonyl-CoA levels. Leptin has also been shown to increase fatty acid oxidation in skeletal muscle through the activation of AMPK. However, we demonstrate that leptin had no significant effect on AMPK activity, AMPK phosphorylation state, acetyl-CoA carboxylase (ACC) activity, or malonyl-CoA levels. AMPK activity and its phosphorylation state were also unaffected after 5 and 10 minutes of perfusion in the presence of leptin. The addition of insulin (100 μU/ml) to the perfusate reduced the ability of leptin to increase fatty acid oxidation and decrease cardiac TG content. These data demonstrate for the first time that leptin activates fatty acid oxidation and decreases TG content in the heart. We also show that the effects of leptin in the heart are independent of changes in the AMPK-ACC-malonyl-CoA axis.

Introduction

In liver, skeletal muscle and pancreas, leptin partitions fatty acids toward fatty acid oxidation rather than TG storage [reviewed in 1]. The mechanism by which leptin increases fatty acid oxidation and decreases TG content in peripheral tissues is not completely understood. Recently, leptin was suggested to increase fatty acid oxidation acutely in skeletal muscle through the activation of AMP-activated protein kinase (AMPK) (2).

AMPK has a key role as a fuel gauge in the heart [reviewed in 3] and plays an important role in regulating fatty acid oxidation (4). The regulation of cardiac fatty acid oxidation by AMPK occurs through the phosphorylation and inhibition of acetyl-CoA carboxylase (ACC) (4). ACC produces malonyl-CoA, which potently inhibits carnitine palmitoyltransferase 1 (CPT-1) (5). Thus the activation of cardiac AMPK reduces ACC activity, lowers malonyl-CoA levels and stimulates fatty acid oxidation (6,7). Furthermore, the activation of AMPK increases fatty acid oxidation and decreases fatty acid incorporation into TG in liver and skeletal muscle (8). Although leptin receptors are present in the heart (9), it is not known whether leptin regulates cardiac fatty acid metabolism or whether leptin can modify the AMPK-ACC-malonyl-CoA axis in the heart.

Fatty acids and glucose are supplied to the heart exogenously from the plasma or endogenously from TG or glycogen stores. Increasing the insulin concentration switches the substrate preference of the heart away from fatty acids and towards glucose utilization (10). Several studies have shown that leptin and insulin signaling pathways interact (11-13) and that AMPK is inhibited by insulin (14-16). Furthermore, leptin has been shown to oppose the effects of insulin on fatty acid partitioning in muscle (17).

The aim of this study was to delineate whether leptin directly affects cardiac fatty acid metabolism and mechanical function in isolated working rat hearts. We further investigated the potential role of the AMPK-ACC-malonyl-CoA axis in the regulation of cardiac fatty acid metabolism by leptin. Lastly, we investigated whether the presence of insulin influenced leptin action on cardiac fatty acid metabolism.

Methods

Leptin Dose

Circulating plasma leptin levels in lean rats are between 2-5 ng/ml. In obesity, plasma leptin levels are elevated to 20-60 ng/ml, depending on the extent of obesity. In this study, we used a concentration of 60 ng/ml leptin to determine the effect of leptin on cardiac function and metabolism. Although this concentration is considered pathophysiological, many studies have used even higher doses to investigate leptin action. Furthermore, leptin has been shown to bind fatty acids in the plasma, although the functional significance of the binding is yet unknown (18). Since our perfusate contains 0.8 mM palmitate, we used 60 ng/ml leptin to ensure that the leptin was not completely bound to fatty acids in the perfusate.

Heart Perfusions

Isolated working hearts from male Sprague-Dawley rats were subjected to aerobic perfusion with a modified Krebs-Henseleit solution containing 5 mM glucose, 0.8 mM palmitate, 3% bovine serum albumin, 2.5 mM free Ca^{2+}, in the absence or presence of 100 μU/ml insulin. Spontaneously beating hearts were perfused at an 11.5 mmHg left atrial preload and an 80 mmHg aortic afterload. Functional parameters were measured as described in Chapter 2.

The hearts, in which fatty acid oxidation and glucose oxidation rates were measured, were subjected to a 60-minute perfusion with perfusate containing [9,10-^{3}H]palmitate and [1-^{14}C]glucose. Leptin (60 ng/ml or 3.8 nmol/l) or vehicle was added 5 minutes into the perfusion period. Palmitate and glucose oxidation rates were determined as described in Chapter 2. Acetyl-CoA production was calculated as described in Chapter 2. Cardiac efficiency was defined as the ratio of cardiac work to the total acetyl-CoA produced.

Shorter perfusion protocols were also performed under identical perfusate conditions as above. The perfusion protocol consisted of a 20-minute period of aerobic perfusion to allow for stabilization followed by the addition of leptin (60 ng/ml or 3.8 nmol/l) or vehicle and a subsequent 5 or 10-minute perfusion period. Hearts were then rapidly frozen at the end of the 5 or 10-minute perfusion for biochemical analysis.

[^{3}H]Palmitate Incorporation into Triacylglycerol

To determine palmitate incorporation into TG, an aliquot of the total cardiac TG was resuspended in 4 ml scintillation fluid and then counted for the presence of radiolabelled [^{3}H]palmitate. Total (exogenous and endogenous) fatty acid oxidation was determined as described in Chapter 2.

Tissue Extractions

Tissue TG levels were determined according to the method of Folch as described in Chapter 2. CoA esters were extracted and subjected to HPLC analysis as described in Chapter 2. ACC activity was performed on 6% PEG fractions as described in Chapter 2. 6% PEG fractions were used to determine AMPK activity with the AMARA peptide as described in Chapter 2.

Immunoblotting

Whole cell homogenates were used for immunoblotting with phospho-AMPK (Thr172) antibody (Cell Signaling Technology®) for determination of phosphorylated AMPK as described in Chapter 2.

Statistical Analysis

All data are presented as the mean ± standard error (n). The data were analyzed with the statistical program Instat 2.01®. The unpaired Student t-test was used to evaluate the statistical significance of differences between the control and leptin-treated groups. Two-tailed values of $p<0.05$ were considered significant.

The effect of leptin (60 ng/ml or 3.8 nmol/l) on cardiac function in isolated working rat hearts subjected to a 60-minute period of aerobic perfusion is shown in Table 3-1. This concentration of leptin had no significant effect on heart rate, peak systolic pressure, cardiac output or cardiac work compared with control hearts. Heart function was stable in both groups throughout the perfusion period and did not differ in any parameter between 5 and 60 minutes of perfusion (data not shown).

The cumulative production of ^3H_2O increased linearly over the 60-minute perfusion period in both the control and leptin-treated hearts. As demonstrated in Fig. 3-1A, leptin significantly increased steady-state palmitate oxidation rates 60% above control levels. Total palmitate uptake ([^{3}H]palmitate oxidized + [^{3}H]palmitate incorporated into TG) into the heart was also significantly increased in the leptin-treated hearts compared with the control hearts (99 ± 7 (n=7) vs. 68 ± 5 (n=6) μmol · g dry wt^{-1}). However, leptin had no effect on glucose oxidation rates in these hearts (Fig. 3-1B).

Leptin also significantly reduced cardiac TG content to 75% the level of control hearts by the end of the 60-minute perfusion period (Fig. 3-2A). In order to investigate whether the leptin-induced decrease in TG content was due to decreased incorporation of fatty acid into TG or increased TG lipolysis, we also determined the amount of [^{3}H]palmitate incorporated into the cardiac TG pool during the perfusion (Fig. 3-2B). Leptin had no significant effect on the incorporation of [^{3}H]palmitate into TG compared with control hearts, suggesting that the leptin-induced decrease in TG content was due to an increased rate of TG lipolysis.

Previous studies have demonstrated that myocardial TG lipolysis provides an important source of fatty acids for oxidative metabolism (19). Since leptin treatment reduced TG content, total fatty acid oxidation rates must take into account the exogenous palmitate oxidized as well as the endogenous fatty acid released from TG. Thus, we calculated total fatty acid oxidation using the following equation: [total [^{3}H]palmitate label incorporation into TG during the perfusion] + [the change in fatty acid content in TG during the perfusion] + [total [^{3}H]palmitate oxidized during the perfusion]. This calculation makes the following assumptions: 1) the TG pool size was similar in control and leptin-treated hearts at the onset of leptin administration, 2) the TG pool size remains constant in the control hearts throughout the perfusion period, which we have shown to occur under these perfusion conditions (19), 3) the amount of [^{3}H]palmitate label entering the TG pool equals that of endogenous or unlabelled fatty acid leaving the pool and 4) all palmitate released from the TG pool is oxidized. Previous studies of TG turnover and the contribution of TG to energy substrate utilization in isolated working hearts demonstrate that these assumptions are valid (19,20). Using the above calculation to determine total fatty acid oxidation, we show that hearts exposed to leptin have total fatty acid oxidation rates that were 82% above control hearts (Fig. 3-3A)

The contribution of glucose and both exogenous and endogenous palmitate to acetyl-CoA production is shown in Fig. 3-3B. Under the perfusion conditions used, fatty acids were the predominant source of acetyl-CoA in both control and leptin-treated hearts. Leptin treatment resulted in an increase in total acetyl-CoA production. This increase in acetyl-CoA production was specifically due to an increase in fatty acid oxidation, with no change in the contribution from glucose metabolism. The leptin-treated hearts also consumed significantly more oxygen than the control hearts (Fig. 3-4A). Although cardiac work was unchanged between control and leptin-treated hearts, the leptin-treated hearts exhibited a significant decrease in cardiac efficiency (i.e. cardiac work/ acetyl-CoA produced) (Fig. 3-4B).

The leptin-induced activation of fatty acid oxidation in skeletal muscle has recently been suggested to be mediated via activation of AMPK (2). However, as demonstrated in Fig. 3-5, the leptin-induced activation of fatty acid oxidation in the heart was not accompanied by changes in AMPK activity measured in the absence of AMP. The maximally stimulatable AMPK activity, measured in the presence of 200 μM AMP, was also not significantly different between control and leptin-treated hearts (1427 ± 92 (n=7) vs. 1244 ± 202 (n=6) pmol · min $^{-1}$· mg protein $^{-1}$. The presence of 200 μM AMP resulted in a 1.3-fold increase in AMPK activity compared with activity measured in the absence of AMP in both the control and leptin-treated hearts. We also determined the level of Thr-172 phosphorylation of AMPK, which is indicative of AMPK activation through its upstream kinase, AMPKK (21). No difference in the level of Thr-172 phosphorylation of AMPK was observed between control and leptin-treated hearts (Fig. 3-5). Representative immunoblots demonstrate variable levels of Thr-172 phosphorylation in the individual hearts, which correlates directly with the level of AMPK activity.

Previous studies have shown that leptin signaling can occur rapidly within minutes (11-13). To determine whether leptin may have resulted in an earlier activation of AMPK, which may have been reversed by the end of the 60-minute perfusion period, we determined whether leptin could activate AMPK at earlier time points. However, after exposure of hearts to leptin for 5 minutes (Fig. 3-6A) or 10 minutes (Fig. 3-6B), we were still unable to show any significant difference in AMPK activity measured in the absence of AMP or Thr-172 phosphorylation of AMPK. The maximally stimulatable AMPK activity, measured in the presence of 200 μM AMP, was also not significantly different between control and 5 minute leptin-treated hearts (902 ± 283 (n=3) vs. 1376 ± 178 (n=6) pmol $\cdot$ min $^{-1}$ $\cdot$ mg protein $^{-1}$. The presence of 200 μM AMP resulted in a 1.8-fold increase in AMPK activity compared with activity measured in the absence of AMP in both the control and 5 minute leptin-treated hearts. Furthermore, the maximally stimulatable AMPK activity, measured in the presence of 200 μM AMP, was also not significantly different between control and 10 minute leptin-treated hearts

(1315 ± 143 (n=4) vs. 1188 ± 55 (n=6) pmol · min $^{-1}$ · mg protein $^{-1}$. The presence of 200 μM AMP resulted in a 2-fold increase in AMPK activity compared with activity measured in the absence of AMP in both the control and 10 minute leptin-treated hearts.

We also determined if leptin had any effects on cardiac ACC activity or malonyl-CoA levels, both of which are established downstream targets of AMPK action (4-7). Leptin had no significant effect on cardiac ACC activity measured in the absence of citrate or malonyl-CoA levels (Table 3-2). ACC activity, measured in the presence of 10 mM citrate, resulted in a 2.5-fold increase in ACC activity compared with ACC activity measured in the absence of citrate in both control and leptin-treated hearts. Maximally stimulatable ACC activity was also not significant between the control and leptin-treated hearts (7.3 ± 0.5 (n=7) vs. 8.5 ± 0.7 (n=6) nmol · min $^{-1}$ · mg protein $^{-1}$). Increased ratios of AMP:ATP are well known to activate AMPK, but neither AMP (1.4 ± 0.3 (n=7) vs. 1.4 ± 0.3 (n=6) μmol · g dry wt $^{-1}$) nor ATP (9.4 ± 0.9 (n=7) vs. 10.3 ± 1.3 (n=6) μmol · g dry wt $^{-1}$) levels were different between control and leptin-treated hearts.

Lastly, we investigated whether 100 μU/ml insulin (600 pmol/l) had any effect on leptin action in the isolated working rat heart. In hearts perfused with leptin + insulin, no significant effect on the mechanical function was observed compared with hearts perfused with Krebs-Henseleit solution containing insulin (Table 3-3). However, insulin did reduce the leptin-induced activation of exogenous fatty acid oxidation (Table 3-4). Furthermore, the leptin-induced depletion of cardiac TG was also blocked by insulin (Table 3-4). While the presence of insulin significantly increased glucose oxidation rates relative to hearts perfused in the absence of insulin (954 ± 51 (n=7) compared with 448 ± 51 (n=6) nmol $[^{14}C]$glucose · g dry wt $^{-1}$ · min $^{-1}$), glucose oxidative rates were not significantly different between insulin and leptin + insulin hearts (Table 3-4). Lastly we also demonstrate that in hearts perfused with leptin + insulin, there was no difference in AMPK activity measured in the absence of AMP, ACC activity measured in the absence of citrate, or malonyl-CoA levels compared with insulin alone (Table 3-4). The maximally stimulatable AMPK activity, measured in the presence of 200 μM AMP, was also not significantly different between insulin and insulin + leptin-treated hearts (1886 ± 208 (n=7) vs. 1687 ± 207 (n=6) pmol · min $^{-1}$ · mg protein $^{-1}$. The presence of 200 μM AMP resulted in a 1.8-fold increase in AMPK activity compared with activity measured in the absence of AMP in both the insulin and insulin + leptin-treated hearts. ACC activity, measured in the presence of 10 mM citrate, resulted in a 1.5-fold increase in ACC activity compared with ACC activity measured in the absence of citrate in both insulin and insulin + leptin-treated hearts. Maximally stimulatable ACC activity was also not significant between the insulin and insulin + leptin-treated hearts (10.7 ± 0.9 (n=6) vs. 7.8 ± 1.2 (n=5) nmol · min $^{-1}$ · mg protein $^{-1}$).

TABLE 3-1.

The Effect of Leptin on Mechanical Function of Isolated Working Rat Hearts

	Control (n=7)	Leptin (n=6)
Heart Rate (beats · min $^{-1}$)	228 ± 10	244 ± 8
Peak Systolic Pressure (mmHg)	128 ± 8	118 ± 3
HR x PSP (beats · min $^{-1}$ · mmHg $^{-1}$ · 10^{-3})	29 ± 1	29 ± 1
Cardiac Output (ml · min $^{-1}$)	54 ± 3	60 ± 4
Cardiac Work (ml · mmHg · min $^{-1}$ · 10^{-2})	69 ± 5	71 ± 5

Values are means ± SE (n). Hearts were perfused with Krebs-Henseleit solution containing 5 mM glucose, 0.8 mM palmitate, and 2.5 mM free Ca^{2+} in the absence or presence of 60 ng/ml leptin. Hearts were subjected to 11.5 mmHg preload and 80 mmHg afterload. HR X PSP = heart rate X peak systolic pressure.

TABLE 3-2.

The Effect of Leptin on Cardiac ACC Activity and Malonyl-CoA Levels

	Control (n=7)	Leptin (n=6)
ACC Activity (nmol $\cdot$ min^{-1} $\cdot$ mg^{-1})	3.3 ± 0.5	3.6 ± 0.5
Malonyl-CoA (nmol $\cdot$ g dry wt^{-1})	3.1 ± 0.7	3.1 ± 0.7

Values are means ± SE (n). ACC activity and malonyl-CoA levels were measured in hearts following the 60-minute perfusion protocol.

TABLE 3-3.

The Effect of Leptin + Insulin on Mechanical Function of Isolated Working Rat Hearts

	Insulin (n=7)	Leptin + Insulin (n=6)
Heart Rate (beats · min $^{-1}$)	239 ± 10	246 ± 12
Peak Systolic Pressure (mmHg)	126 ± 5	117 ± 4
HR x PSP (beats · min $^{-1}$ · mmHg $^{-1}$ · 10^{-3})	30 ± 2	29 ± 2
Cardiac Output (ml · min $^{-1}$)	52 ± 4	53 ± 5
Cardiac Work (ml · mmHg · min $^{-1}$ · 10^{-2})	66 ± 7	62 ± 8

Values are means ± SE (n). Hearts were perfused with Krebs-Henseleit solution containing 5 mM glucose, 0.8 mM palmitate, 100 μU/ml (600 pmol/l) insulin and 2.5 mM free Ca^{2+} in the absence or presence of 60 ng/ml leptin. Hearts were subjected to 11.5 mmHg preload and 80 mmHg afterload. HR X PSP = heart rate X peak systolic pressure.

TABLE 3-4.

The Effect of Leptin + Insulin on Oxidative Rates, TG Content and AMPK-ACC-malonyl-CoA

	Insulin (n=7)	Leptin + Insulin (n=6)
Palmitate Oxidation (nmol ^{3}H palmitate · g dry wt $^{-1}$ · min $^{-1}$)	1108 ± 279	1429 ± 291
Glucose Oxidation (nmol ^{14}C glucose · g dry wt $^{-1}$ · min $^{-1}$)	954 ± 88	1141 ± 249
TG Content (μmol · g dry wt $^{-1}$)	22 ± 2	21 ± 3
[^{3}H]Palmitate Incorporation into TG (μmol · g dry wt $^{-1}$)	6 ± 1	6 ± 1
AMPK Activity (pmol · min^{-1} · mg protein^{-1})	1133 ± 142	941 ± 116
ACC Activity (nmol · min^{-1} · mg protein^{-1})	7.3 ± 0.8	5.9 ± 1.2
Malonyl-CoA (nmol · g dry wt^{-1})	3.8 ± 0.5	3.3 ± 1.1

Values are means ± SE (n) measured in hearts subjected to the 60-minute perfusion period.

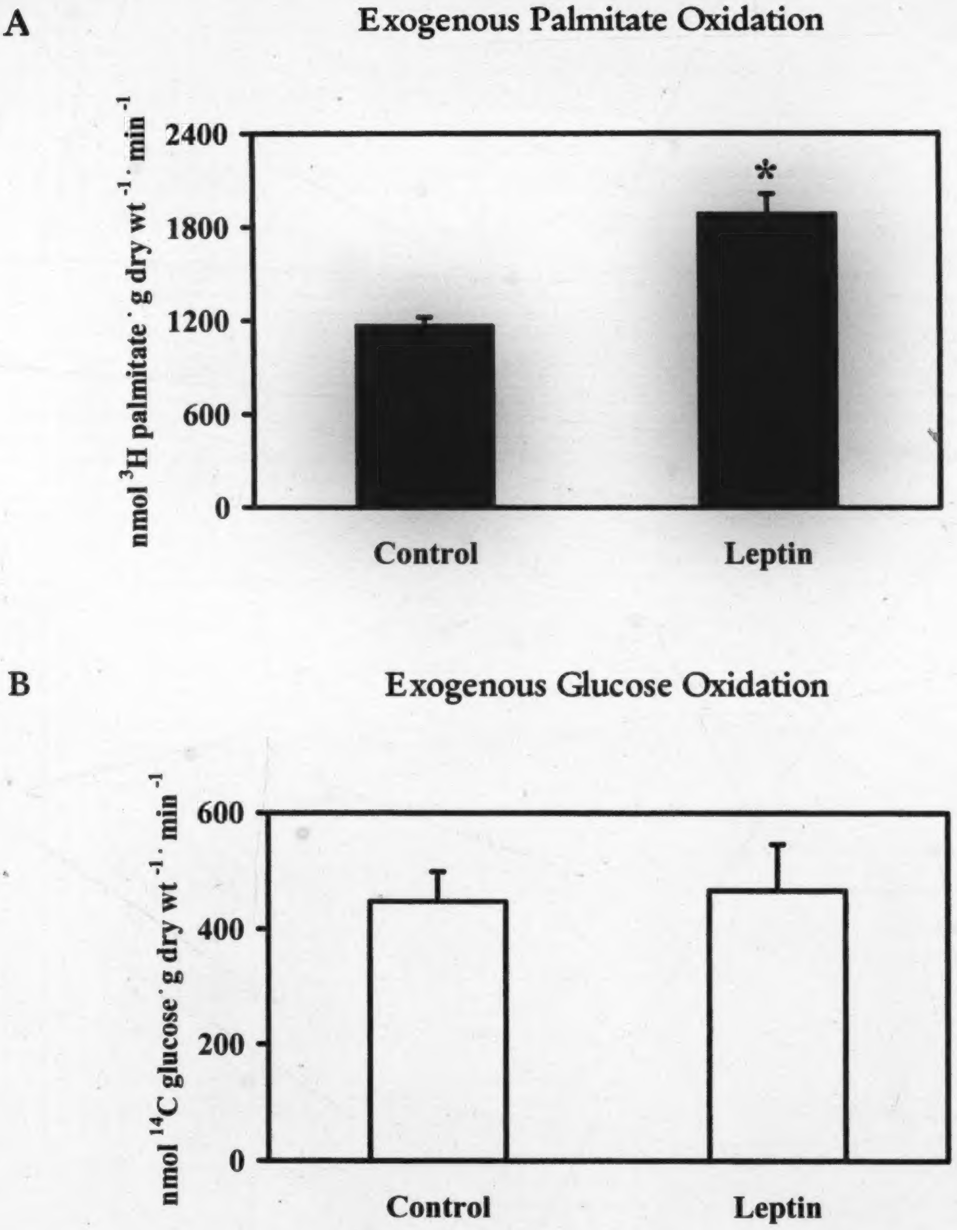


Figure 3-1. The effect of 60 ng/ml leptin on rates of exogenous palmitate (A) and glucose oxidation (B). Values are mean ± SE (n=7 control, n=6 leptin-treated). *Significantly different from control hearts.

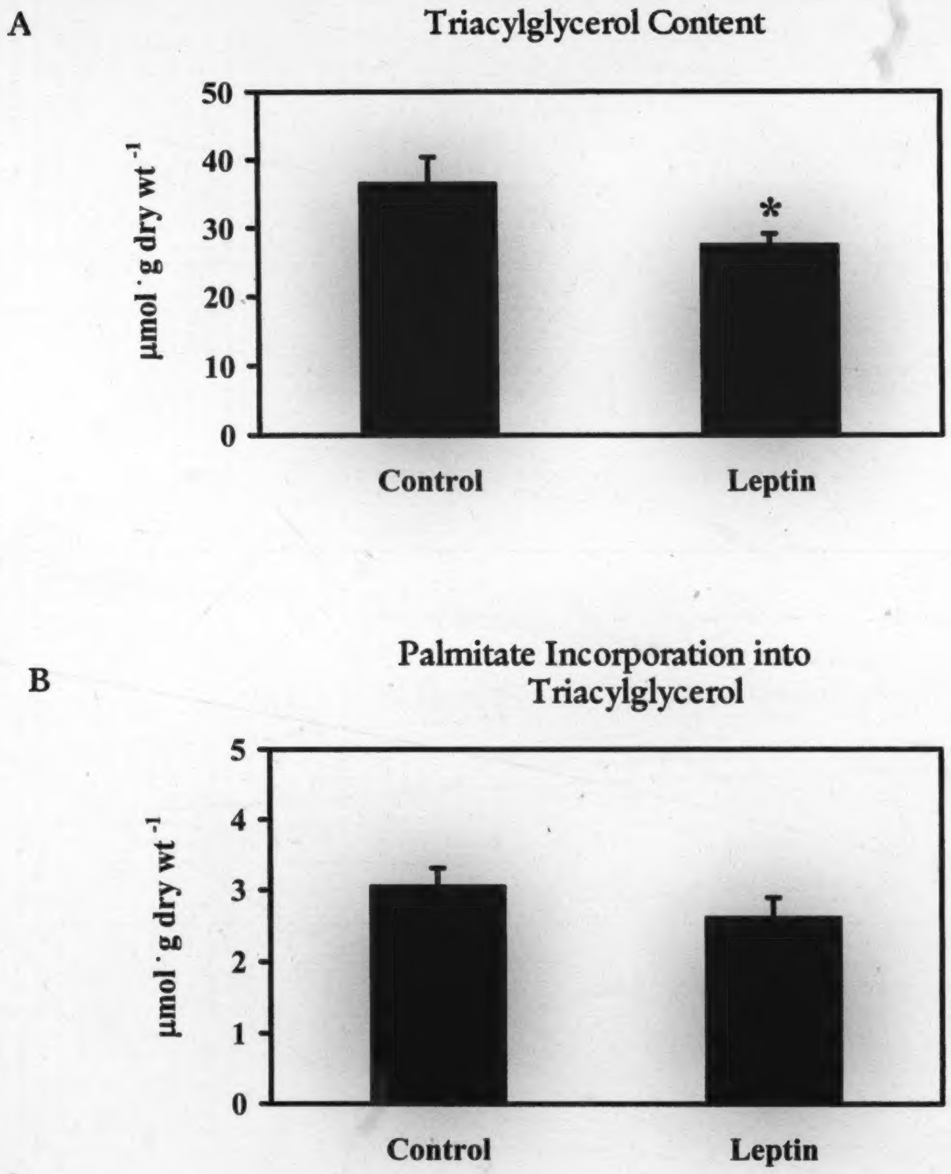


Figure 3-2. The effect of 60 ng/ml leptin on total cardiac triacylglycerol content (A) and palmitate incorporation into triacylglycerol (B). Values were measured in hearts following the 60-minute perfusion period. Values are mean ± SE (n=7 control, n=6 leptin-treated). *Significantly different from control hearts.

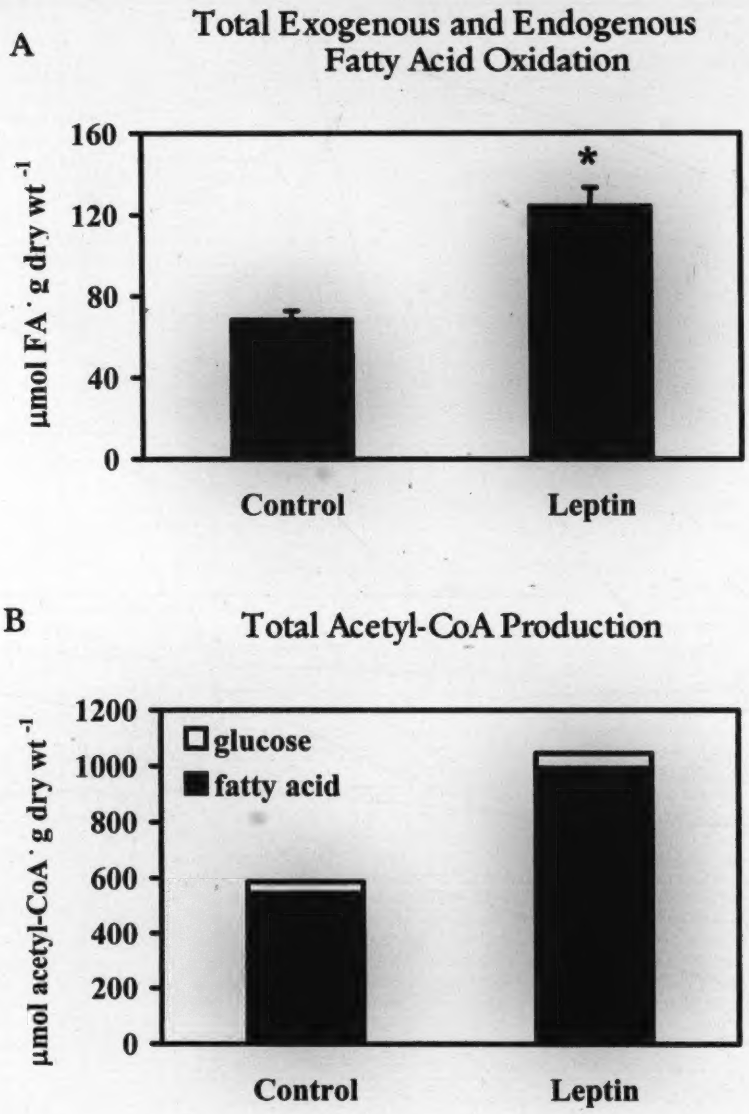


Figure 3-3. The effect of 60 ng/ml leptin on total exogenous and endogenous fatty acid oxidation (A) and total acetyl-CoA production (B). Values were determined as described in Chapter 2. Values are mean ± SE (n=7 control, n=6 leptin-treated). *Significantly different from control hearts.

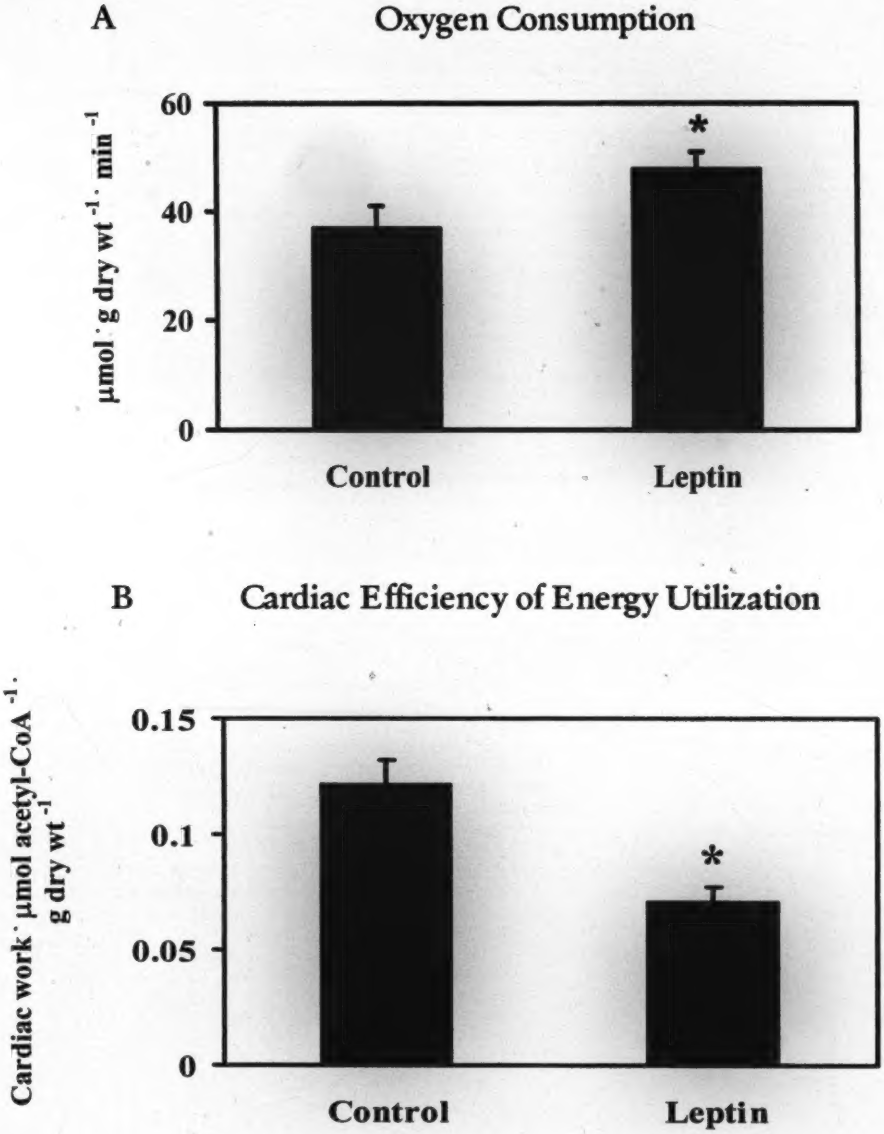


Figure 3-4. The effect of 60 ng/ml leptin on oxygen consumption (A) and the efficiency of cardiac energy production (B). Values were determined as described in "Experimental Procedures". Values are mean ± SE (n=7 control, n=6 leptin-treated). *Significantly different from control hearts.

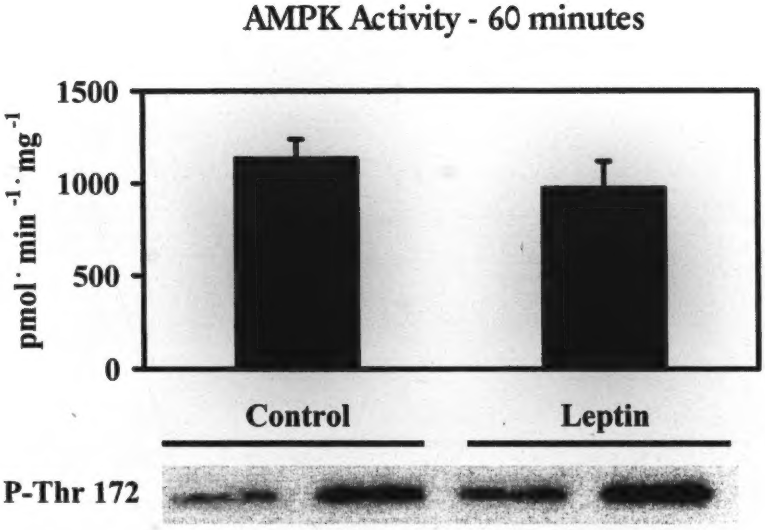


Figure 3-5. The effect of 60 ng/ml leptin on cardiac AMPK activity and Thr-172 phosphorylation of AMPK in hearts exposed to leptin for 60 minutes. Values are mean ± SE (n=7 control, n=6 leptin-treated).

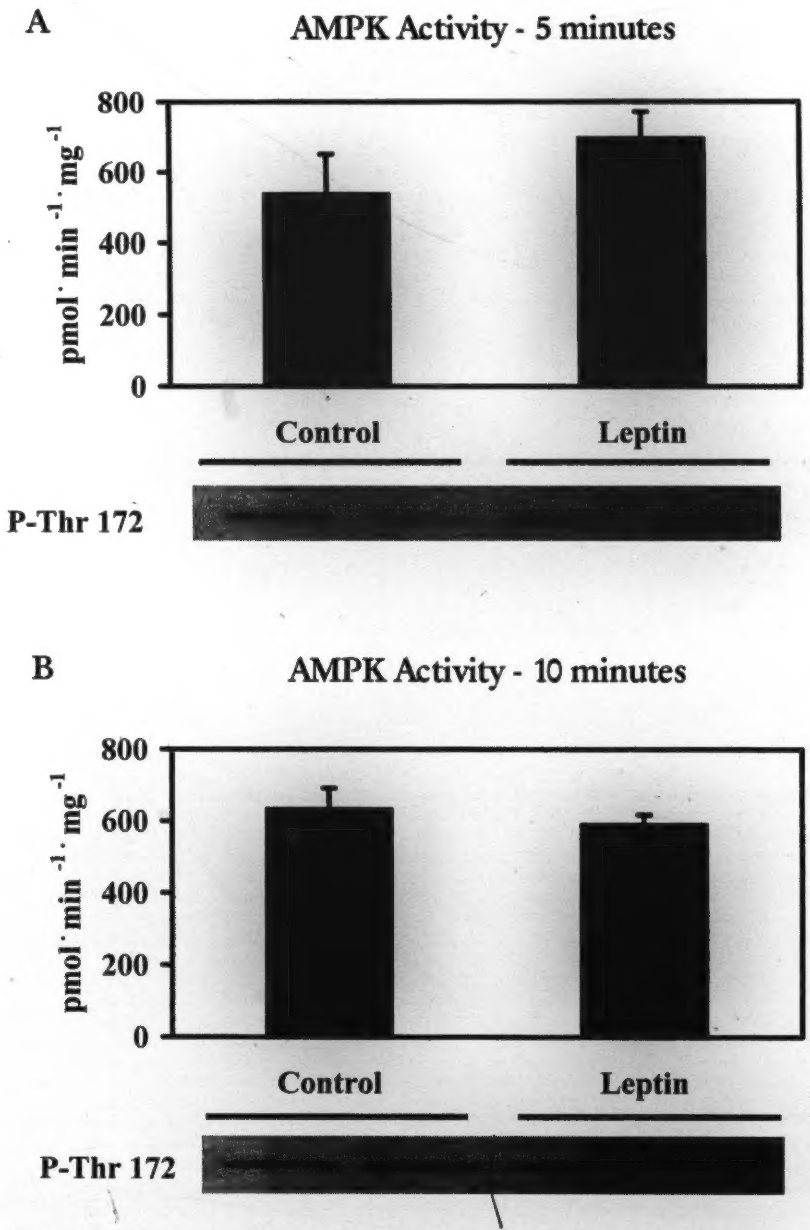


Figure 3-6. The effect of 60 ng/ml leptin on cardiac AMPK activity and Thr-172 phosphorylation of AMPK in hearts exposed to leptin for 5 minutes (A) and 10 minutes (B). Values are mean ± SE (n=7 control, n=6 leptin-treated).

Discussion

This study demonstrates for the first time that leptin significantly alters cardiac energy metabolism by preferentially increasing fatty acid oxidation rates and stimulating TG lipolysis. Leptin also increases cardiac acetyl-CoA production and oxygen consumption in the absence of any effect on contractile function, resulting in a significant reduction in cardiac efficiency. This suggests that leptin can have important actions on cardiac energy production. We also show that the leptin-induced stimulation of cardiac fatty acid oxidation and mitochondrial metabolism does not occur through the activation of the AMPK-ACC-malonyl-CoA axis. Finally, we show that insulin can prevent the leptin-induced stimulation of fatty acid oxidation and TG lipolysis.

Recently AMPK was identified as the signaling cascade through which leptin activates fatty acid oxidation in skeletal muscle (2). However, we demonstrate that leptin had no effect on cardiac AMPK activity or AMPK phosphorylation after either 5, 10 or 60 minutes of leptin exposure. After the 60-minute perfusion period, there was also no change in the downstream mediators involved in the regulation of cardiac FA oxidation: ACC activity or malonyl-CoA levels. This suggests that, in the heart, leptin can regulate fatty acid oxidation independent of AMPK.

The reason why leptin activates AMPK in skeletal muscle and not in heart may be related to differences in AMPK subunit expression and regulation between these tissues. AMPK is a heterotrimeric complex comprising of an α catalytic subunit and two regulatory subunits (β and γ) (22). AMPK can be activated through phosphorylation of Thr-172 by AMPKK (21) and through increases in AMP:ATP (23) and Cr:PCr ratios (24). Several isoforms of each subunit have been identified with varying tissue distribution (25).. The α2β2γ1 and α2β2γ2 complexes are highly expressed in both cardiac and skeletal muscle (22,26). However, the α2β2γ3 complex is exclusively expressed in skeletal muscle (26). In skeletal muscle, direct activation of α2-AMPK by leptin was accompanied by an increase in AMP levels and Thr-172 phosphorylation (2). However, in isolated working rat hearts, we observed no change in AMP levels or Thr-172 phosphorylation. While further investigation is required, it is possible that the activation of AMPK by leptin is specific to skeletal muscle and is mediated through the γ3 isoform. Because isoform specific AMPK activity was not determined in this study, we cannot completely rule out the possibility that α2-AMPK activity was increased by leptin. However, if α2-AMPK was activated, changes in Thr-172 phosphorylation, ACC activity and malonyl-CoA levels would be expected and these changes were not observed.

In the isolated working rat heart, leptin had no significant effect on any measured index of cardiac function (peak systolic pressure, cardiac output or cardiac work). However, leptin treatment increased myocardial fatty acid oxidation 60% above control hearts and decreased TG content by 33%. Studies of triglyceride turnover in the heart have demonstrated that myocardial TG pools undergo substantial turnover ie. continuous lipolysis and synthesis (19). Thus, we determined the amount of [^{3}H]palmitate label in the TG pool and demonstrated that the leptin-induced decrease in TG content was due to an increase in TG lipolysis rather than a decrease in palmitate incorporation into TG. Previous studies have also demonstrated that myocardial TG lipolysis provides an important source of fatty acids for oxidative metabolism (19). We further determined that the total (exogenous and endogenous) fatty acid oxidation in leptin-treated hearts was elevated 82% above control hearts. Thus, we have demonstrated that leptin increases the oxidation of palmitate derived from exogenous sources as well as from the lipolysis of endogenous myocardial TG.

In addition to an increase in fatty acid oxidation, leptin also increased total acetyl-CoA production and oxygen consumption. This resulted in a significant decrease in cardiac efficiency of energy utilization in the leptin-treated hearts. Furthermore, the leptin-induced increase in TCA cycle activity is specifically due to an activation of fatty acid oxidation originating from exogenous palmitate and endogenous TG with no change in the contribution from glucose metabolism. While elevated rates of fatty acid oxidation are associated with reduced cardiac efficiency, they may be important in preventing the 'lipotoxicity' or toxic effects associated with TG accumulation (27,28). Indeed, in the heart, TG accumulation is associated with depressed contractile function, arrhythmias, hypertrophy, heart failure and apoptosis (29-34). The reduction of myocardial TG content by troglitazone treatment results in the normalization of cardiac function (29). Thus, elevated leptin levels in obesity may play a protective role in limiting TG accumulation and its associated cardiac dysfunction. However, the potential anti-steatotic benefit of leptin in the heart must be weighed against the elevated rates of fatty acid oxidation and decrease in cardiac efficiency.

Several studies have shown that leptin and insulin signaling pathways interact (11-13) and further that AMPK can be activated by leptin (2) and inhibited by insulin (14-16). In this study we demonstrate that the leptin-induced activation of fatty acid oxidation and decrease in TG content is reduced by insulin. We also show that acute exposure to leptin does not interfere with the ability of insulin to stimulate glucose metabolism in the heart. The mechanism by which insulin inhibited the actions of leptin is not clear. However, our data do show that insulin reduced the leptin-induced activation of fatty acid oxidation and TG depletion without changes in AMPK activity, ACC activity or malonyl-CoA levels.

Leptin has also been shown to induce reactive oxygen species generation by increasing fatty acid oxidation in aortic endothelial cells through the activation of protein kinase A (PKA), which results in the phosphorylation of ACC and a reduction of ACC activity and malonyl-CoA levels (35). It is therefore possible that leptin may be acting through PKA activation. However the elevation of cAMP levels in the heart is associated with increases in contractile function that were not observed in the presence of leptin. Furthermore, there was no change in ACC activity or malonyl-CoA levels. Thus, although PKA activity was not measured, it seems that leptin does not mediate the activation of fatty acid oxidation in the heart through this mechanism.

Summary

In conclusion, this study demonstrated for the first time that leptin specifically alters cardiac metabolism by preferentially increasing fatty acid oxidation and TG lipolysis. This occurs independently of changes in the AMPK-ACC-malonyl-CoA axis. Leptin also increased myocardial oxygen consumption and significantly reduced cardiac efficiency. Given that elevated rates of fatty acid oxidation and decreased cardiac efficiency can contribute to contractile dysfunction and cardiovascular disease, future studies are clearly needed to establish whether leptin is indeed the link between obesity and cardiovascular disease.

References

---

1. Hynes GR, Jones PJ: Leptin and its role in lipid metabolism. *Curr Opin Lipidol* 12:321-327, 2001

2. Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, Kahn BB: Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 415:339-343, 2002

3. Hardie DG, Carling D: The AMP-activated protein kinase—fuel gauge of the mammalian cell? *Eur J Biochem* 246:259-273, 1997

4. Saddik M, Gamble J, Witters LA, Lopaschuk GD: Acetyl-CoA carboxylase regulation of fatty acid oxidation in the heart. *J Biol Chem* 268:25836-25845, 1993

5. McGarry JD, Stark MJ, and Foster DW: Hepatic malonyl-CoA levels of fed, fasted and diabetic rats as measured using a simple radioisotopic assay. *J Biol Chem* 253:8291-8293, 1978

6. Kudo N, Gillespie JG, Kung L, Witters LA, Schulz R, Clanachan AS, Lopaschuk GD: Characterization of 5'AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. *Biochim Biophys Acta* 1301:67-75, 1996

7. Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD: High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *J Biol Chem* 270:17513-17520, 1995

8. Muoio DM, Seefeld K, Witters LA, Coleman RA: AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target. *Biochem J* 338:783-791, 1999

9. Tartaglia LA: The leptin receptor. *J Biol Chem* 272:6093-6096, 1997

10. Neely JR, Morgan HE: Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. *Ann Rev Physiol* 36:415-439, 1974

11. Carvalheira JB, Siloto RM, Ignacchitti I, Brenelli SL, Carvalho CR, Leite A, Velloso LA, Gontijo JA, Saad MJ: Insulin modulates leptin-induced STAT3 activation in rat hypothalamus. *FEBS Lett* 500:119-124, 2001

12. Szanto I, Kahn CR: Selective interaction between leptin and insulin signaling pathways in a hepatic cell line. *Proc Natl Acad Sci USA* 97:2355-2360, 2000

13. Kim YB, Uotani S, Pierroz DD, Flier JS, Kahn BB: *In vivo* administration of leptin activates signal transduction directly in insulin-sensitive tissues: overlapping but distinct pathways from insulin. *Endocrinology* 141:2328-2339, 2000

14. Witters LA, Kemp BE: Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase. *J Biol Chem* 267:2864-2867, 1992

15. Gamble J, Lopaschuk GD: Insulin inhibition of 5' adenosine monophosphate-activated protein kinase in the heart results in activation of acetyl coenzyme A carboxylase and inhibition of fatty acid oxidation. *Metabolism* 46:1270-1274, 1997

16. Beauloye C, Marsin AS, Bertrand L, Krause U, Hardie DG, Vanoverschelde JL, Hue L: Insulin antagonizes AMP-activated protein kinase activation by ischemia or anoxia in rat hearts, without affecting total adenine nucleotides. *FEBS Lett* 505:348-352, 2001

17. Muoio DM, Dohm GL, Tapscott EB, Coleman RA: Leptin opposes insulin's effects on fatty acid partitioning in muscles isolated from obese ob/ob mice. *Am J Physiol* 276:E913-E921, 1999

18. Campbell FM, Gordon MJ, Hoggard N, Dutta-Roy AK: Interaction of free fatty acids with human leptin. *Biochem Biophys Res Commun* 247:654-658, 1998

19. Saddik M, Lopaschuk GD: Myocardial triglyceride turnover and contribution to energy substrate utilization in isolated working rat hearts. *J Biol Chem* 266:8162-8170, 1991

20. Saddik M, Lopaschuk GD: Myocardial triglyceride turnover during reperfusion of isolated rat hearts subjected to a transient period of global ischemia. *J Biol Chem* 267:3825-3831, 1992

21. Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG: Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J Biol Chem* 271:27879-27887, 1996

22. Davies SP, Hawley SA, Woods A, Carling D, Haystead TA, Hardie DG: Purification of the AMP-activated protein kinase on ATP-gamma-sepharose and analysis of its subunit structure. *Eur J Biochem* 223:351-357, 1994

23. Moore F, Weekes J, Hardie DG: Evidence that AMP triggers phosphorylation as well as direct allosteric activation of rat liver AMP-activated protein kinase. A sensitive mechanism to protect the cell against ATP depletion. *Eur J Biochem* 199:691-697, 1991

24. Ponticos M, Lu QL, Morgan JE, Hardie DG, Partridge TA, Carling D: Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *EMBO J* 17:1688-1699, 1998

25. Woods A, Cheung PC, Smith FC, Davison MD, Scott J, Beri RK, Carling D: Characterization of AMP-activated protein kinase beta and gamma subunits. Assembly of the heterotrimeric complex in vitro. *J Biol Chem* 271:10282-10290, 1996

26. Cheung PC, Salt IP, Davies SP, Hardie DG, Carling D: Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. *Biochem J* 346:659-669, 2000

27. Shimabukuro M, Koyama K, Chen G, Wang MY, Trieu F, Lee Y, Newgard CB, Unger RH: Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc Natl Acad Sci USA* 94:4637-4641, 1997

28. Unger RH: Lipotoxic diseases. *Annu Rev Med* 53:319-336, 2002

29. Zhou YT, Grayburn P, Karim A, Shimabukuro M, Higa M, Baetens D, Orci L, and Unger RH: Lipotoxic heart disease in obese rats: implications for human obesity. *Proc Natl Acad Sci USA*. 97:1784-1789, 2000

30. Chiu HC, Kovacs A, Ford DA, Hsu FF, Garcia R, Herrero P, Saffitz JE, Schaffer JE: A novel mouse model of lipotoxic cardiomyopathy. *J Clin Invest* 107:813-822, 2001

31. Corr PB, Creer MH, Yamada KA, Saffitz JE, Sobel BE: Prophylaxis of early ventricular fibrillation by inhibition of acylcarnitine accumulation. *J Clin Invest* 83:927-936, 1989

32. Regan TJ, Weisse AB: Diabetic cardiomyopathy. *J Am Coll Cardiol* 19:1165-1166, 1992

33. Zoneraich S, Mollura JL: Diabetes and the heart: state of the art in the 1990's. *Can J Cardiol* 9:293-299, 1993

34. Fiordaliso F, Latini R, Li B, Malhotra A, and Kajstura J: Myocyte loss in the diabetic heart is mediated by apoptosis. *Circulation* 100(Suppl I):759, 1999

35. Yamagishi SI, Edelstein D, Du XL, Kaneda Y, Guzman M, Brownlee M: Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. *J Biol Chem* 276:25096-25100, 2001

CHAPTER 4.

Potential Mechanisms and Consequences of Cardiac Triacylglycerol Accumulation in Insulin Resistant Rats

---

A version of this chapter has been published. Atkinson LL, Kozak R, Kelly SE, Onay-Besikci A, Russell JC, Lopaschuk GD. *Am J Physiol* [epub ahead of print] Dec 3, 2002

My role in this work involved performing all the biochemical experiments (except those noted below) as well as writing the manuscript. Ray Kozak performed the heart perfusions. Arzu Onay-Besikci performed the CPT-I activity measurements. Ken Strynadka performed the HPLC analysis of CoA esters.

CHAPTER 4.

Potential Mechanisms and Consequences of Cardiac Triacylglycerol Accumulation in Insulin Resistant Rats

Abstract

The accumulation of intracellular triacylglycerol (TG) is highly correlated with muscle insulin resistance. However it is controversial whether the accumulation of TG is the result of increased fatty acid supply, decreased fatty acid oxidation, or both. It is also not clear whether increases in TG synthesis or decreases in TG lipolysis play a role in the accumulation of muscle TG. Since abnormal fatty acid metabolism is a key contributor to the pathogenesis of diabetes-related cardiovascular dysfunction, we examined fatty acid and glucose metabolism in hearts of insulin resistant JCR:LA-cp rats. Isolated working hearts from insulin resistant rats had glycolytic rates that were reduced to 50% of lean control levels ($p<0.05$). Cardiac TG content was increased by 50% ($p<0.05$) in the insulin resistant rats but palmitate oxidation rates remained similar among the insulin resistant and lean control rats. However, plasma fatty acids and TG levels, as well as cytosolic heart type fatty acid binding protein (H-FABP) protein

expression, were significantly increased in the insulin resistant rats. AMP-activated protein kinase (AMPK) plays a major role in the regulation of cardiac fatty acid and glucose metabolism. When activated, AMPK increases fatty acid oxidation by inhibiting acetyl-CoA carboxylase (ACC) and reducing malonyl-CoA levels and decreases TG content by inhibiting glycerol-3-phosphate acyltransferase (GPAT), the first committed step in TG synthesis. The activation of AMPK also stimulates cardiac glucose uptake and glycolysis. We thus investigated whether a decrease in AMPK activity was associated with a reduced cardiac glycolysis and increased TG content in the insulin resistant rats. However, we found no significant difference in AMPK activity. We also found no significant difference in various established downstream targets of AMPK: ACC activity, malonyl-CoA levels, CPT-1 activity, or GPAT activity. We conclude that hearts from insulin resistant JCR:LA-cp rats accumulate substantial TG as a result of increased fatty acid supply rather than reduced fatty acid oxidation. Furthermore, the accumulation of cardiac TG is associated with a reduction in insulin-stimulated glucose metabolism.

Introduction

The insulin resistant syndrome is a pre-type 2 diabetic state characterized by hyperinsulinemia and impaired insulin-stimulated glucose metabolism [reviewed in 1]. In muscle, the accumulation of intracellular triacylglycerol (TG) is highly correlated with the development of insulin resistance, as well as the severity of insulin resistance [reviewed in 2]. However, it remains controversial whether the accumulation of TG in muscle is the result of increased fatty acid supply, decreased fatty acid oxidation, or a combination of both (2). It is also not clear whether increased TG synthesis or decreased TG lipolysis play a role in TG accumulation.

Under normal physiological conditions, the energy requirements of the heart are fulfilled primarily by the oxidation of fatty acids with the remainder provided by carbohydrate metabolism (3). Several studies have implicated abnormal fatty acid metabolism as being an important contributor to the pathogenesis of diabetes-related cardiovascular dysfunction. For example, in untreated type 1 diabetes, elevated plasma fatty acid levels result in a decreased utilization of glucose and an over-reliance of the heart on fatty acid oxidation [reviewed in 4]. Similarly, increased rates of cardiac fatty acid oxidation have been demonstrated in db/db mice, a model of type 2 diabetes (5).

An important enzyme controlling both fatty acid and glucose metabolism is AMP-activated protein kinase (AMPK). AMPK acts as a metabolic sensor or "fuel gauge" in the mammalian cell and regulates key proteins controlling fatty acid oxidation, TG synthesis, glucose uptake [reviewed in 6] and glycolysis (7). The activation of cardiac AMPK reduces ACC activity, lowers malonyl-CoA levels and stimulates fatty acid oxidation (8,9). The activation of AMPK also results in the phosphorylation and inhibition of glycerol-3-phosphate acyltransferase (GPAT), the committed step in *de novo* synthesis of TG (10). Thus, through the coordinated regulation of key enzymes in fatty acid metabolism, AMPK regulates the flux of fatty acids into oxidation rather than TG storage (10).

The male JCR:LA-cp rat is a model of pre-type 2 diabetes that displays complete skeletal muscle insulin resistance by the age of 12 weeks (11). In this study, we investigated fatty acid and glucose metabolism in isolated working rat hearts from 12 week old insulin resistant male JCR:LA-cp rats. We further investigated whether changes in AMPK activity were associated with the altered fatty acid and glucose metabolism in the insulin resistant hearts.

Animals

The study was performed on male insulin resistant (cp/cp) and lean (either +/cp or +/+) male rats at 12 weeks of age. Rats were handled twice a week from birth to ensure miminal stress at the time of sacrifice. At 12 weeks of age, male rats were fasted overnight (unless specified) and anesthetized without restraint in a large glass jar with 3% halothane at 1 l/min O_2.

Five different groups of 12 week old insulin resistant and lean control rats were used in this study. This included rats used for: 1) blood collection 2) isolated working heart perfusions: rats fasted overnight prior to experiment 3) isolated working heart perfusions: rats fed prior to experiment 4) obtaining frozen heart tissue and 5) fresh hearts used for isolation of mitochondria.

Plasma Measurements

One group of non-fasted insulin resistant rats and a group of lean control rats were anesthetized as above and cardiac puncture was performed to obtain a 6 ml blood sample. Samples were analyzed for plasma glucose, insulin, free fatty acids, and TG levels as described in Chapter 2.

Heart Perfusions

Isolated working hearts from fasted lean and insulin resistant JCR:LA-cp rats were subjected to an aerobic perfusion with a modified Krebs-Henseleit solution containing 0.8 mM palmitate, 5 mM glucose, 3% bovine serum albumin, 2.5 mM free Ca^{2+}, and 500 μU/ml (3000 pmol/l) insulin. This perfusion condition is referred to as 'high fat/low glucose/low insulin'. Spontaneously beating hearts were perfused at an 11.5 mm Hg left atrial preload and an 80 mm Hg aortic afterload. Functional parameters were measured as described in Chapter 2.

The hearts, in which fatty acid oxidation and glycolytic rates were measured, were subjected to a 40-minute perfusion with perfusate containing [1-^{14}C]palmitate and D-[5-^{3}H]glucose (12). Palmitate oxidation and glycolytic rates were determined as described in Chapter 2. At the end of the perfusions, heart ventricles were quickly frozen with Wollenberger clamps cooled to the temperature of liquid N_2.

In another series of perfusions, hearts were taken from non-fasted (fed) JCR:LA-cp rats and perfused with Krebs-Henseleit solution containing 0.4 mM palmitate, 11 mM glucose, and 2000 μU/ml (12000 pmol/l) insulin to mimic plasma levels of fatty acids, glucose and insulin of fed rats. This perfusion condition is referred to as 'low fat/high glucose/high insulin'. Palmitate oxidation was determined as described in Chapter 2.

Tissue Collection

One group of fed insulin resistant and a group of lean control rats were anesthetized as above. Once surgical plane was reached, hearts were rapidly isolated, frozen immediately in liquid N_2 and stored at $-80^{\circ}C$ until analysis. Visible adipose tissue was quickly dissected from the heart prior to freezing.

Tissue Extractions

Tissue triacylglycerol levels were determined using silicic acid separation as described in Chapter 2. CoA esters were extracted and subjected to HPLC analysis as described in Chapter 2. ACC, GPAT and CPT-1 activities were performed as described in Chapter 2. AMPK activity was performed using the AMARA peptide as described in Chapter 2.

Immunoblotting

Whole cell homogenates were used for immunoblotting with anti-H-FABP (SantaCruz Biotechnology) for determination of protein expression as described in Chapter 2.

Statistical Analysis

All data are presented as the mean ± standard error (n). The data were analyzed with the statistical program Instat 2.01®. Unpaired student t-tests were used to determine the statistical significance of differences between the insulin resistant JCR:LA-cp and lean control rats. Two-tailed values of $p<0.05$ were considered significant.

Results

Table 4-1 depicts some characteristics of 12 week old insulin resistant JCR:LA-cp and lean control rats. Body weights were significantly greater in insulin resistant rats compared with lean control rats. In addition, the insulin resistant rats had greater heart weights than the lean control rats. The relative increase in body weight, however, exceeded that of the increase in heart mass, resulting in no significant difference in the heart weight to body weight ratio in the insulin resistant rats compared with the lean controls. Fasting plasma insulin levels were significantly elevated in the insulin resistant rats compared with the lean controls, demonstrating the presence of insulin resistance. However, the insulin resistant rats remain normoglycemic as indicated by similar fasting plasma glucose levels.

Mechanical function was monitored continuously over the 40-minute perfusion period in the spontaneously beating isolated working hearts. All hearts were subject to a constant left atrial preload of 11.5 mmHg and an aortic afterload of 80 mmHg. As shown in Table 4-2, heart rate was slightly but non-significantly lower in the insulin resistant hearts compared with lean control hearts, whereas no differences were evident in peak systolic pressure or cardiac output. Both coronary and aortic flows were also continuously monitored which allowed for the determination of cardiac work (the product of cardiac output and peak systolic pressure) as an index of mechanical function. There was no significant difference in cardiac work (Table 4-2) between the insulin resistant and lean control hearts.

Isolated working hearts from fasted insulin resistant rats were perfused with Krebs-Henseleit solution containing 0.8 mM palmitate, 5 mM glucose and 500 μU/ml (3000 pmol/l) insulin. This perfusion condition is termed 'high fat/low glucose/low insulin'. As demonstrated in Fig. 4-1, glycolytic rates in the insulin resistant hearts were reduced to 50% of lean control values. However, there was no significant difference in palmitate oxidation rates in the insulin resistant rat hearts compared with the lean controls (Fig. 4-2A). We further determined palmitate oxidation rates in hearts from fed insulin resistant rats with Krebs-Henseleit solution containing 0.4 mM palmitate, 11 mM glucose, 2000 μU/ml (12000 pmol/l) insulin. This perfusion condition is termed 'low fat/high glucose/high insulin'. Fatty acid oxidation rates in the lean rat hearts perfused under low fat/high glucose/high insulin conditions were lower than rates determined in the lean rat hearts perfused under high fat/low glucose/low insulin conditions, but did not reach statistical significance (compare Fig. 4-2B to Fig. 4-2A). Similarly, we found no significant difference in palmitate oxidation rates between fed lean and fed insulin resistant rat hearts (Fig. 4-2B).

In obesity-related insulin resistance, the metabolic capacity of skeletal muscle appears to be organized toward TG storage rather than oxidation (2). Furthermore, the accumulation of TG is strongly associated with reduced muscle insulin sensitivity (2). Thus, we next determined whether TG content was increased in insulin resistant rat hearts. Fig. 4-3 demonstrates that the insulin resistant rats have a 2-fold increase in cardiac TG content compared with lean controls.

The accumulation of TG can be the result of increased fatty acid uptake, increased de novo fatty acid synthesis or decreased fatty acid oxidation rates. Since de novo fatty acid synthesis does not play an important role in the heart and we observed no difference in fatty acid oxidation rates, we next investigated whether cardiac fatty acid supply was increased. Table 4-3 demonstrates that plasma fatty acid and TG levels were significantly elevated in the insulin resistant rats compared with lean controls. Furthermore, cytosolic H-FABP protein expression was also increased in the insulin resistant rats compared with lean controls. These data are all consistent with an increased fatty acid supply to the insulin resistant rat hearts compared with the lean controls.

The AMPK signaling cascade plays a key role in the regulation of fatty acid oxidation, TG synthesis and glucose metabolism (6,7,10). We therefore determined whether a decrease in AMPK activity was associated with the decreased glycolysis and elevated TG content in the insulin resistant rat hearts. Fig. 4-4 demonstrates that there was a slight but non-significant decrease in AMPK activity measured in the absence of AMP in the insulin resistant hearts. The maximally stimulatable AMPK activity, measured in the presence of 200 μM AMP, was also not significantly different between lean and insulin resistant hearts (514 ± 89 (n=8) vs. 432 ± 60 (n=8) pmol $\cdot$ min $^{-1\cdot}$ mg protein $^{-1}$. The presence of

200 μM AMP resulted in a 2.5-fold increase in AMPK activity compared with activity measured in the absence of AMP in both the lean and insulin resistant hearts. We further investigated whether key proteins downstream of AMPK involved in the regulation of fatty acid oxidation and TG synthesis were altered. However, in the insulin resistant rat hearts, there was no difference in ACC activity measured in the absence of citrate compared with lean controls (Fig. 4-5A). ACC activity, measured in the presence of 10 mM citrate, resulted in a 2.4-fold increase in ACC activity compared with ACC activity measured in the absence of citrate in the lean hearts and resulted in a 3.3-fold increase in ACC activity compared with ACC activity measured in the absence of citrate in the insulin resistant hearts. Maximally stimulatable ACC activity was significantly increased in the insulin resistant hearts compared with the lean hearts (18.8 ± 1.0 (n=8) vs. 12.9 ± 1.5 (n=8) nmol · min $^{-1}$ · mg protein $^{-1}$, $p<0.05$). Furthermore, malonyl-CoA levels (Fig. 4-5B) and CPT-1 activity (Fig. 4-6A) remained unchanged in the insulin resistant rat hearts compared with lean controls.

AMPK also plays a role in the regulation of TG synthesis (10). As demonstrated in Fig. 4-6B, there was a slight but non-significant decrease in mitochondrial GPAT activity in the insulin resistant hearts compared with the lean controls. The microsomal GPAT activity in the insulin resistant hearts was also not significantly different between the insulin resistant and lean hearts (77 ± 6 (n=6) vs. 100 ± 9 (n=5) pmol · min $^{-1}$ ·mg protein $^{-1}$). Interestingly, total GPAT activity (mitochondrial + microsomal) was significantly decreased in the insulin resistant compared with lean hearts (97 ± 9 (n=6) vs. 129 ± 10 (n=5) pmol $\cdot$ min $^{-1}$ $\cdot$mg protein $^{-1}$, $p<0.05$).

TABLE 4-1.

Characteristics of 12 week lean and insulin resistant rats

	Lean (n=5)	Insulin Resistant (n=6)
Heart Weight (grams)	1.4 ± 0.1	1.5 ± 0.1
Body Weight (grams)	295 ± 7	390 ± 12*
Heart Weight:Body Weight Ratio (X 1000)	4.6 ± 0.3	3.8 ± 0.3
Plasma Insulin (pmol/l)	272 ± 27	2746 ± 439*
Plasma Glucose (mmol/l)	9.5 ± 0.4	10.1 ± 0.7

Values are means ± SE (n). * Significantly different from lean controls.

TABLE 4-2.

Mechanical function of isolated working rat hearts from 12 week old lean and insulin resistant rats

	Lean (n=5)	Insulin Resistant (n=6)
Heart Rate (beats · min $^{-1}$)	263 ± 7	229 ± 15
Peak Systolic Pressure (mmHg)	102 ± 2	106 ± 2
HR x PSP (beats · min $^{-1}$ · mmHg $^{-1}$ · 10^{-3})	27 ± 1	24 ± 1
Cardiac Output (ml · min $^{-1}$)	46 ± 6	48 ± 7
Cardiac Work (ml · mmHg · min $^{-1}$ · 10^{-2})	47 ± 7	51 ± 7

Values are means ± SE (n). Hearts were perfused with Krebs-Henseleit solution containing 5 mM glucose, 0.8 mM palmitate, 3% albumin, and 500 μU/ml (3000 pmol/l) insulin. Hearts were subjected to a 11.5 mmHg left atrial preload and 80 mmHg aortic afterload. HR X PSP = heart rate X peak systolic pressure.

TABLE 4-3.

Plasma lipid levels and cytosolic H-FABP protein expression in 12 week old lean and insulin resistant rats

	Lean (n=5)	Insulin Resistant (n=6)
Plasma fatty acids (mmol/l)	0.27 ± 0.02	0.37 ± 0.06*
Plasma triacylglycerol (mmol/l)	1.76 ± 0.15	6.44 ± 0.54*
Heart-FABP protein expression (relative units)	0.91 ± 0.03	1.08 ± 0.02*

Values are means ± SE (n). Plasma fatty acid and triacylglycerol measurements were performed on samples obtained from fed lean and insulin resistant rats. Heart-FABP protein expression was determined using immunoblotting and densitometric analysis as described in Chapter 2. * Significantly different from lean controls.

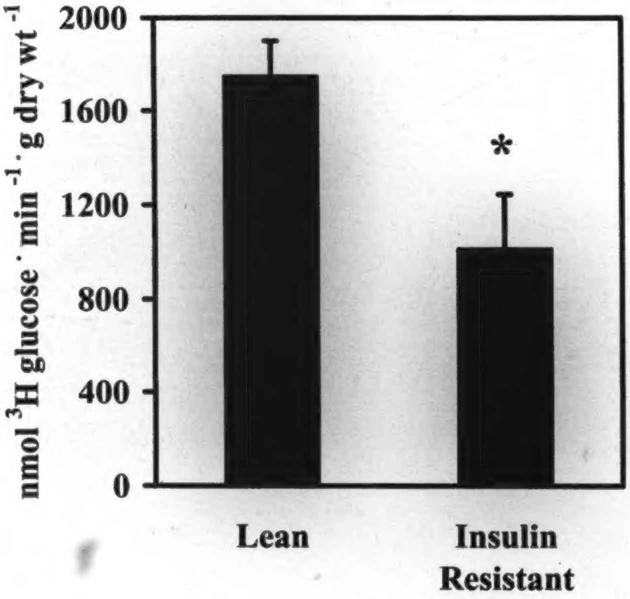


Figure 4-1. Glycolytic rates in isolated working hearts from lean and insulin resistant rats. Hearts were perfused with 0.8 mM palmitate, 5 mM glucose, and 500 μU/ml (3000 pmol/l) insulin, as described in Chapter 2. Values are means ± SE (n=5 lean, n=6 insulin resistant). *Significantly different from lean controls.

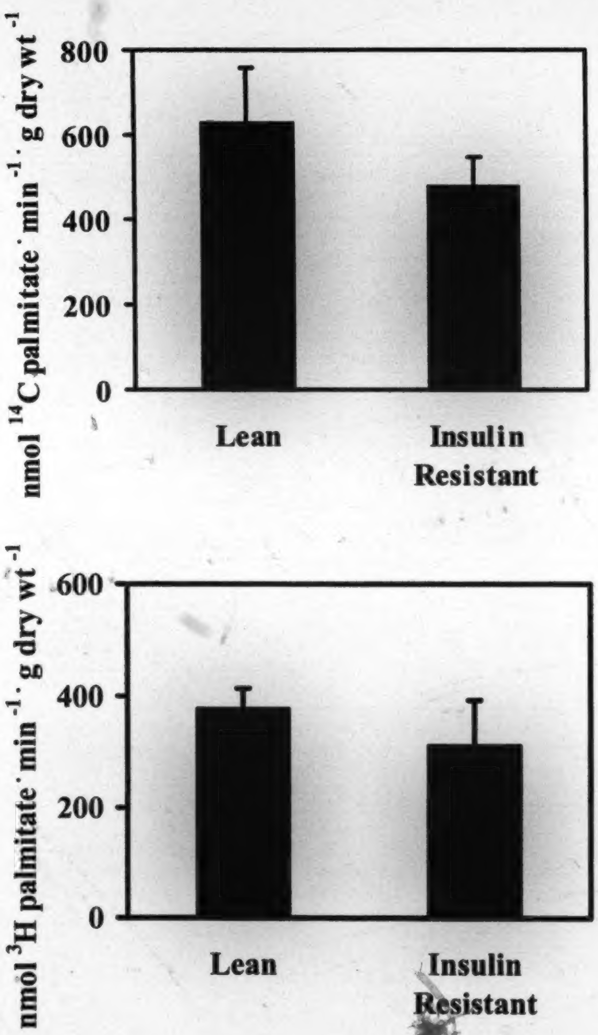


Figure 4-2. Palmitate oxidation rates in isolated working hearts from lean and insulin resistant rats under fasting (A) and fed (B) conditions. Hearts were from either fasted or fed animals. Hearts were perfused under 'high fat/low glucose/low insulin' for fasted and under 'low fat/high glucose/high insulin' for fed as described in Chapter 2. Values are means ± SE (n=5 lean fasting, n=6 insulin resistant fasting, n=6 lean fed, n=5 insulin resistant fed).

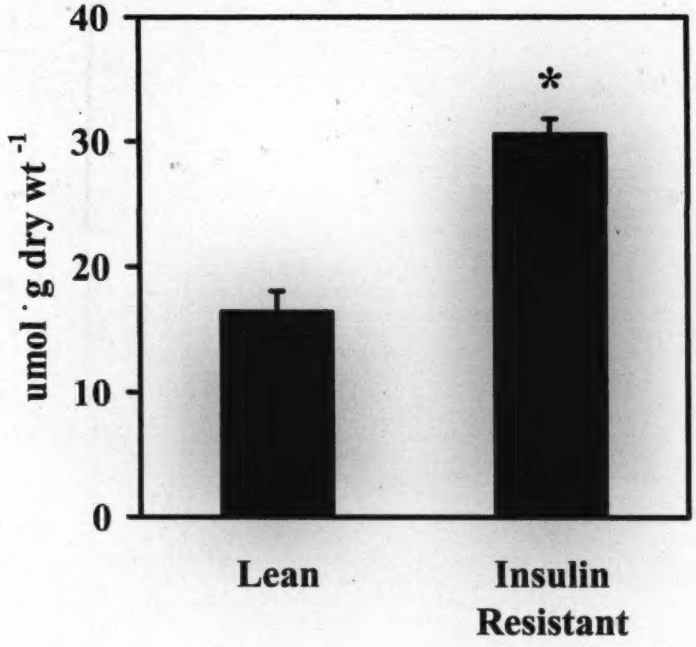


Figure 4-3. Cardiac triacylglycerol content in lean and insulin resistant rats. Values are mean ± SE (n=6). *Significantly different from lean controls.

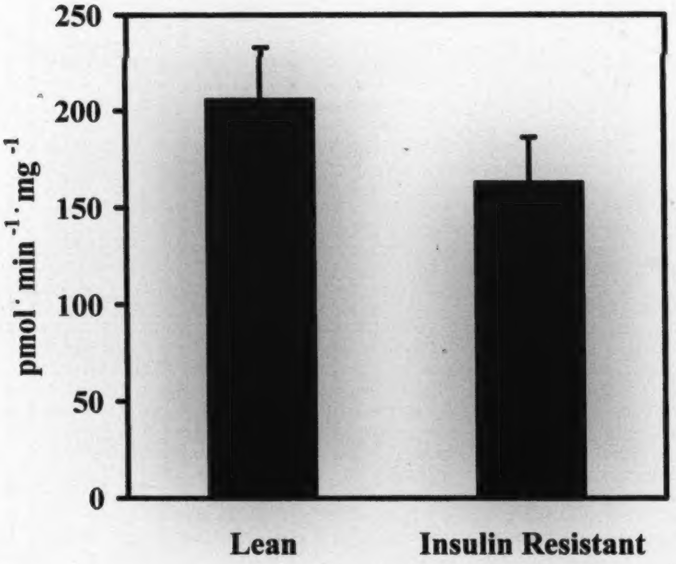


Figure 4-4. Cardiac AMPK activity in lean and insulin resistant rats. Values are means ± SE (n=8).

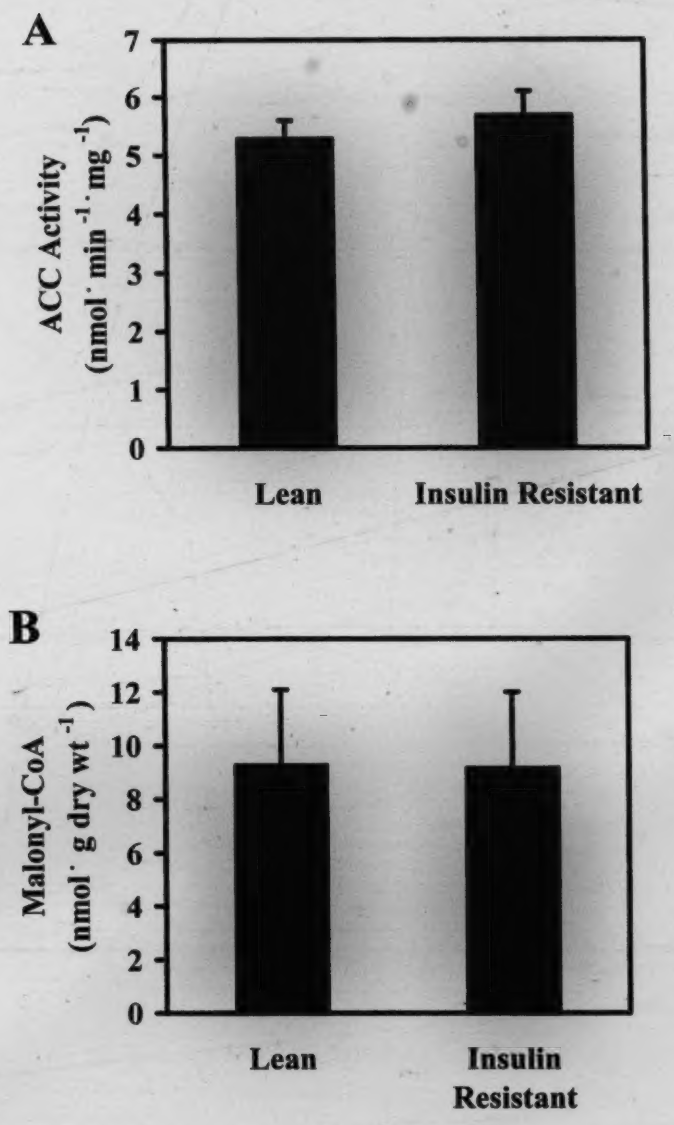


Figure 4-5. Cardiac ACC activity (A) and malonyl-CoA levels (B) in lean and insulin resistant rats. Values are means ± SE (n=4 lean ACC activity, n=7 insulin resistant ACC activity, n=6 lean malonyl-CoA, n=5 insulin resistant malonyl-CoA).

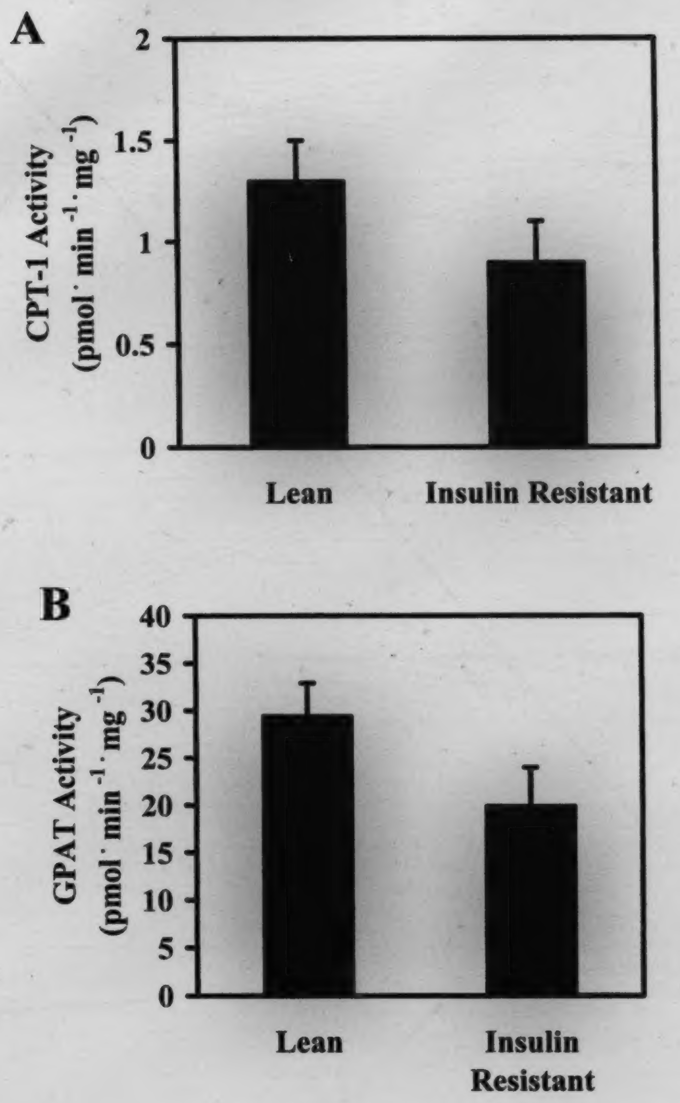


Figure 4-6. Cardiac CPT-1 activity (A) and mitochondrial GPAT activity (B) in lean and insulin resistant rats. Values are means ± SE (n=5 lean, n=6 insulin resistant).

Discussion

In this study, we demonstrate that hearts from insulin resistant JCR:LA-cp rats have a substantial accumulation of TG. We further demonstrate that this appears to be the result of an increased supply and uptake of myocardial fatty acids as opposed to a decrease in fatty acid oxidation rates or increase in TG synthesis. The increase in intracellular TG content was also associated with a significant decrease in insulin-stimulated glycolysis. However, although the substrate preference of the hearts was dramatically altered, there was no significant difference in AMPK activity, a key regulator of glycolysis, fatty acid oxidation and TG content.

In this study, we used isolated working hearts from insulin resistant JCR:LA-cp rats to investigate potential alterations in cardiac fatty acid and glucose metabolism. We demonstrate that the insulin resistant rat hearts have a 50% reduction in glycolytic rates compared with lean controls. This observation is in agreement with studies in the obese Zucker rat, another rodent model of insulin resistance, which also show that cardiac glycolytic rates are reduced by approximately 50% (12). We further demonstrate that the cardiac TG content was significantly increased in the insulin resistant hearts compared with lean controls. In contrast, there was no significant difference in palmitate oxidation rates between the insulin resistant and lean control hearts perfused under either 'high fat/low glucose/low insulin' or 'low fat/high glucose/high insulin' conditions.

The accumulation of intracellular TG is strongly associated with the presence of skeletal muscle insulin resistance (2). In this study, we demonstrate a 2-fold increase in the cardiac TG content of insulin resistant rats. The 50% increase in cardiac TG content was directly proportional and associated with the 50% decrease in glycolytic rates. While these data only provides correlative evidence to the relationship of cardiac TG content and reduced glucose utilization, several studies in skeletal muscle have demonstrated this same relationship (13-15). Importantly, previous studies have demonstrated that elevated muscle TG in the JCR:LA-cp rat is strongly associated with the onset of insulin resistance (16). Furthermore, treatment of insulin resistant rats with the lipid-lowering agent, MEDICA 16, prevents the accumulation of muscle TG along with the development of insulin resistance (11,16).

The accumulation of TG could result from increased fatty acid uptake, decreased fatty acid oxidation, or both. Increases in TG synthesis or decreases in TG lipolysis may also play a role in the accumulation of muscle TG. Since fatty acid oxidation rates and microsomal or mitochondrial GPAT activity in the isolated working hearts were not significantly different, we determined whether the supply and uptake of fatty acids into the insulin resistant rat heart was increased. Indeed, plasma fatty acid and TG levels were significantly elevated in the insulin resistant rats indicating that there is an increased supply of fatty acids to the heart. Furthermore, cytosolic H-FABP protein expression was significantly higher in the insulin resistant rats compared with the lean controls suggesting that the insulin resistant rat hearts are also capable of increased fatty acid uptake. This is consistent with studies demonstrating that skeletal muscle from high-fat fed rats accumulates TG as a result of an enhanced ability of the muscle to take up fatty acids (17). Although we have not directly measured fatty acid uptake in the present study, our data are consistent with studies demonstrating both increased cardiac fatty acid uptake and increased FABP mRNA expression in obese Zucker rat hearts (18,19).

AMPK is considered a novel therapeutic target for the treatment of obesity, insulin resistance and type 2 diabetes (20-22) because of its key role in the regulation of glucose and fatty acid metabolism (6). However, although there was an apparent trend for AMPK activity to decrease in the insulin resistant rat hearts, we were unable to demonstrate significant changes in AMPK activity. We also did not observe any relationship between AMPK activity and the increased TG content and decreased glycolysis observed in the insulin resistant rat hearts. Several studies have suggested that AMPK is inhibited by insulin (23-25) and by high glucose in pancreatic beta cells (26). In hyperglycemic db/db mouse hearts, we have observed a significant reduction in AMPK activity suggesting that hyperglycemia may be a prerequisite for the down regulation of AMPK activity (unpublished observation, LL Atkinson, D Severson, GD Lopaschuk).

We also measured the activities of enzymes involved in the control of fatty acid oxidation and TG synthesis: ACC activity, CPT-1 activity and GPAT activity. However, we were unable to demonstrate any significant difference in these key enzymes that are established downstream targets of AMPK. Thus, these data suggest that alterations in AMPK do not mediate the primary alterations occurring in cardiac substrate metabolism in insulin resistance. However, one may speculate that reductions in AMPK activity result when hyperglycemia and type 2 diabetes develops. Further studies are required to elucidate potential changes in AMPK activity that may occur in type 2 diabetes.

In a recent study, Young et al. investigated whether impairments in fatty acid oxidation result in cardiac TG accumulation and contractile dysfunction in obese Zucker rats (27). They demonstrate that the obese Zucker rat heart is unable to elevate fatty acid oxidation in response to increased fatty acid availability (27). Consistent with the observations in the obese Zucker rat heart, we demonstrate that the JCR:LA-cp rat hearts have an increased fatty acid supply as well as elevated TG content. However, unlike the obese Zucker rat heart, we find that the JCR:LA-cp rat heart has no difference in fatty acid oxidation rates under different nutritional and perfusion conditions.

One possible explanation for the differences may relate to the age of the animals used and the relative level of TG accumulation. For instance, studies in 7 and 14-week old obese Zucker hearts have demonstrated the presence of TG accumulation without changes in cardiac function (28). However, the continued accumulation of TG eventually results in depressed cardiac function by 20 weeks of age in obese Zucker hearts (28). At this age, troglitazone treatment leads to a reduction in myocardial TG content and the normalization of cardiac function (28) suggesting that there may be a threshold level of TG accumulation that appears to be age-dependent.

Based on body weight, the obese Zucker animals in the study by Young et al. (27) appear to be older than those used in the present study. Furthermore, the obese Zucker rat hearts exhibited contractile dysfunction while the JCR:LA-cp rat hearts had no difference in mechanical function or cardiac work. While the absolute levels of cardiac TG in the obese Zucker heart cannot be compared with the level in the JCR:LA-cp heart due to differences in experimental determination, it may be that the initial accumulation of TG in the insulin resistant heart results from an elevated supply of fatty acids to the heart. Subsequently, impairments in fatty acid oxidation lead to the continued accumulation of TG and contractile dysfunction. While purely speculative, these data collectively present an interesting hypothesis that requires further investigation.

Summary

In summary, we have demonstrated that insulin resistant JCR:LA-cp rat hearts have a dysregulation in fatty acid metabolism whereby substantial TG accumulates due to increased fatty acid supply with no change in fatty acid oxidation rates. Whether the accumulation of cardiac TG plays a role in the development of cardiovascular disease remains to be answered. However, it is interesting to note that the lowering of plasma lipids which should reduce cardiac TG accumulation results in protection against the development of cardiovascular disease in the insulin resistant JCR:LA-cp rat (29,30).

References

---

1. Reaven GM: Role of insulin resistance in human disease (syndrome X): an expanded definition. *Annu Rev Med* 44:121-131, 1993

2. Lewis GF, Carpentier A, Khosrow A, Giacca A: Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. *Endocrine Rev* 23:201-229, 2002

3. Neely JR, Morgan HE: Relationship between carbohydrate and lipid metabolism and the energy balance of the heart muscle. *Ann Rev Physiol* 36:415-439, 1974

4. Lopaschuk GD: Metabolic abnormalities in the diabetic heart. *Heart Fail Rev* 7:149-159, 2002

5. Belke DD, Larsen TS, Gibbs EM, Severson DL: Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (db/db) mice. *Am J Physiol* 279:E1104-E1113, 2000

6. Hardie DG, Carling D: The AMP-activated protein kinase: fuel gauge of the mammalian cell? *Eur J Biochem* 246:259-273, 1997

7. Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L: Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischemia. *Curr Biol* 10:1247-1255, 2000

8. Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD: High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *J Biol Chem* 270:17513-17520, 1995

9. Kudo N, Gillespie JG, Kung L, Witters LA, Schulz R, Clanachan AS, Lopaschuk GD: Characterization of 5'AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. *Biochim Biophys Acta* 1301:67-75, 1996

10. Muoio DM, Seefeld K, Witters LA, Coleman RA: AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target. *Biochem J* 338:783-791, 1999

11. Russell JC, Shillabeer G, Bar-Tana J, Lau DCW, Richardson M, Wenzel LM, Graham SE, Dolphin PJ: Development of insulin resistance in the JCR:LA-cp rat: Role of triacylglycerols and effects of MEDICA 16. *Diabetes* 47:770-778, 1998

12. Morabito D, Montessuit C, Rosenblatt-Velin N, Lerch R, Vallotton MB, Lang U: Impaired glucose metabolism in the heart of obese Zucker rats after treatment with phorbol ester. *Int J Obes Relat Metab Disord* 26:327-334, 2002

13. Pan DA, Lillioja S, Kriketos AD, Milner MR, Baur LA, Bogardus C, Jenkins AB, Storlein LH: Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* 46:983-988, 1997

14. Perseghin G, Scifo P, De Cobelli F, Pagliato E, Battezzati A, Arcelloni C, Vanzulli A, Testolin G, Pozza G, Del Mashie A, Lazy L: Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: A ^{1}H-^{13}C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes* 48:1600-1606, 1999

15. Szczepaniak LS, Babcock EE, Schick F, Dobbins RL, Garg A, Burns DK, McGarry JD, Stein DT: Measurement of intracellular triglyceride stores by ^{1}H spectroscopy: validation in vivo. *Am J Physiol* 276:E977-E989, 1999

16. Atkinson LL, Kelly SE, Russell JC, Bar-Tana J, Lopaschuk GD: MEDICA 16 inhibits hepatic acetyl-CoA carboxylase and reduces plasma triacylglycerol levels in insulin-resistant JCR:LA-cp rats. *Diabetes* 51:1548-1555, 2002

17. Hegarty BD, Cooney GJ, Kraegen EW, Furler SM: Increased efficiency of fatty acid uptake contributes to lipid accumulation in skeletal muscle of high fat-fed insulin-resistant rats. *Diabetes* 51:1477-1484, 2002

18. Berk PD, Zhou SL, Kiang CL, Stump D, Bradbury M, Isola LM: Uptake of long chain free fatty acids is selectively up-regulated in adipocytes of Zucker rats with genetic obesity and non-insulin-dependent diabetes mellitus. *J Biol Chem* 272:8830-8835, 1997

19. Luiken JJ, Arumugam Y, Dyck DJ, Bell RC, Pelsers MM, Turcotte LP, Tandon NN, Glatz JF, Bonen A: Increased rates of fatty acid uptake and plasmalemmal fatty acid transporters in obese Zucker rats. *J Biol Chem* 276:40567-40573, 2001

20. Moller DE: New drug targets for type 2 diabetes and the metabolic syndrome. *Nature* 414:821-827, 2001

21. Winder WW: AMP-activated protein kinase: possible target for treatment of type 2 diabetes. *Diabetes Technol Ther* 2:441-448, 2000

22. Winder WW, Hardie DG: AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* 277:E1-E10, 1999

23. Beauloye C, Marsin AS, Bertrand L, Krause U, Hardie DG, Vanoverschelde JL, Hue L: Insulin antagonizes AMP-activated protein kinase activation by ischemia or anoxia in rat hearts, without affecting total adenine nucleotides. *FEBS Lett* 505:348-352, 2001

24. Gamble J, Lopaschuk GD: Insulin inhibition of 5' adenosine monophosphate-activated protein kinase in the heart results in activation of acetyl coenzyme A carboxylase and inhibition of fatty acid oxidation. *Metabolism* 46:1270-1274, 1997

25. Witters LA, Kemp BE: Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase. *J Biol Chem* 267:2864-2867, 1992

26. Salt IP, Johnson G, Ashcroft SJ, Hardie DG: AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release. *Biochem J* 335:533-539, 1998

27. Young ME, Guthrie PH, Razeghi P, Leighton B, Abbasi S, Patil S, Youker KA, Taegtmeyer H: Impaired long-chain fatty acid oxidation and contractile dysfunction in the obese Zucker rat heart. *Diabetes* 51:2587-2595, 2002

28. Zhou YT, Grayburn P, Karim A, Shimabukuro H, Higa M, Baetens D, Orci L, Unger RH: Lipotoxic heart disease in obese rats: implications for human obesity. *Proc Natl Acad Sci USA* 97:1784-1789, 2000

29. Russell JC, Amy RM, Graham SE, Dolphin PJ, Wood GO, Bar-Tana J: Inhibition of atherosclerosis and myocardial lesions in the JCR:LA-cp rat by beta, beta'-tetramethylhexadecanedioic acid (MEDICA 16). *Artierioscler Thromb Vasc Biol* 15:918-923, 1995

30. Russell JC, Dolphin PJ, Graham SE, Amy RM: Cardioprotective and hypolipidemic effects of nisoldipine in the JCR:LA-cp rat. *J Cardiovasc Pharmacol* 29:586-592, 1997

CHAPTER 5.

MEDICA 16 Inhibits Hepatic Acetyl-CoA Carboxylase and Reduces Plasma Triacylglycerol Levels in Insulin Resistant JCR:LA-cp Rats

A version of this chapter has been published. Atkinson LL, Kelly SE, Bar-Tana J, Russell JC, Lopaschuk GD. *Diabetes* 51:1548-1555, 2002

My role in this work involved performing all the biochemical experiments as well as writing the manuscript.

CHAPTER 5.

MEDICA 16 Inhibits Hepatic Acetyl-CoA Carboxylase and Reduces Plasma Triacylglycerol Levels in Insulin Resistant JCR:LA-cp Rats

Abstract

Intracellular triacylglycerol (TG) content of liver and skeletal muscle contributes to insulin resistance, and a significant correlation exists between TG content and the development of insulin resistance. Since acetyl-CoA carboxylase (ACC) is the rate-limiting enzyme for liver fatty acid biosynthesis and a key regulator of muscle fatty acid oxidation, we examined whether ACC plays a role in the accumulation of intracellular TG. We also determined the potential role of 5'-AMP activated protein kinase (AMPK) in this process, since it can phosphorylate and inhibit ACC activity in both liver and muscle. TG content, ACC and AMPK were examined in the liver and skeletal muscle of insulin resistant JCR:LA-cp rats during the time frame when insulin resistance develops. At 12 weeks of age, there was a 3-fold elevation in liver TG content and a 7-fold elevation in skeletal muscle TG content in the JCR:LA-cp rats compared with lean-age matched controls.

Hepatic ACC activity was significantly elevated in 12-week old JCR:LA-cp rats compared with lean age-matched controls (8.75 ± 0.53 (n=11) vs. 3.30 ± 0.18 (n=10) $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively), even though AMPK activity was also increased. The observed increase in hepatic ACC activity was accompanied by a 300% increase in ACC protein expression. There were no significant differences in ACC activity, ACC protein expression or AMPK activity in the skeletal muscle of the 12-week JCR:LA-cp rats. Treatment of 12-week JCR:LA-cp rats with MEDICA 16 (an ATP-citrate lyase inhibitor) resulted in a decrease in hepatic ACC and AMPK activities, but had no effect on skeletal muscle ACC and AMPK. Our data suggest that alterations in ACC or AMPK activity in muscle do not contribute to the development of insulin resistance. However increased liver ACC activity in the JCR:LA-cp rat appears to contribute to the development of lipid abnormalities, although this increase does not appear to occur secondary to a decrease in AMPK activity.

Introduction

Several studies have implicated a role for intracellular TG in the development of insulin resistance. The lipid content of muscle, liver and pancreas significantly correlates with insulin sensitivity (1,2) and a strong negative correlation has been demonstrated in skeletal muscle between insulin-stimulated glucose uptake and local accumulation of TG (3-6). In support of this, reduction of skeletal muscle, liver and islet TG, with either troglitazone or leptin, results in an increase in insulin-stimulated glucose uptake and a decrease in insulin resistance (6,7).

The accumulation of TG in muscle and liver can either be the result of increased fatty acid uptake, increased *de novo* fatty acid synthesis or decreased fatty acid oxidation. An increase in TG synthesis or decrease in TG lipolysis may also contribute to the accumulation of TG. Both fatty acid synthesis and fatty acid oxidation are controlled by two isoforms of ACC [reviewed in 8]. The 265-kDa isoform of ACC (ACC-265) is the rate-limiting enzyme for fatty acid biosynthesis in liver and adipose tissue [reviewed in 9], since malonyl-CoA produced by ACC-265 is the precursor for fatty acid biosynthesis in liver and adipose tissue. The 280-kDa isoform of ACC (ACC-280) is a major regulator of muscle fatty acid metabolism (10-12), since malonyl-CoA produced by this enzyme is a potent inhibitor of skeletal muscle carnitine palmitoyltransferase 1, a key enzyme involved in mitochondrial fatty acid uptake (13).

Both isoforms of ACC can be regulated at the level of gene expression, allosteric regulation of the enzyme, and by reversible phosphorylation [reviewed in 14,15]. In both liver and muscle, AMP-activated protein kinase (AMPK) is capable of phosphorylating and inhibiting ACC activity (16-18). AMPK acts as a metabolic sensor, and is activated by increases in the AMP:ATP and creatine:phosphocreatine ratios (19,20). Once activated the enzyme switches off ATP-consuming anabolic pathways such as fatty acid synthesis and switches on ATP-producing catabolic pathways such as fatty acid oxidation and glucose uptake. This coordinated control of synthesis and oxidation allows the maintenance of energy balance within the cell. Insulin control of fatty acid biosynthesis and oxidation may also involve AMPK, and in both liver (21) and muscle (22,23), insulin has been shown to inhibit AMPK activity. Whether alterations in insulin regulation of AMPK are altered in insulin resistance is not known.

The JCR:LA-cp male rat develops insulin resistance between the age of 4 and 12 weeks. The cp gene results in a mutation in the leptin receptor resulting in the lack of functional leptin receptors in all tissues of the homozygous JCR:LA-cp rats (24). Studies demonstrating the presence of intracellular lipid droplets in the gastrocnemius muscle of 12-week JCR:LA-cp rats support the concept that TG accumulation in the muscle plays a key role in the development of insulin resistance (25). Of importance is that MEDICA 16, an ATP citrate lyase inhibitor that limits acetyl-CoA supply to ACC, reduces muscle TG content in these insulin-resistant rats (25).

The purpose of this study was to determine the involvement of ACC and AMPK in the accumulation of TG in the liver and skeletal muscle of the JCR:LA-cp insulin resistant rat. This was studied in JCR:LA-cp rats during the critical period in which insulin-resistance develops (4-12 weeks following birth). We also determined whether MEDICA 16 treatment affected either liver and skeletal muscle ACC or AMPK activity in these animals.

Methods

Animals

This study involved the use of obese JCR:LA-cp (cp/cp) and lean age-matched controls (either +/cp or +/+), at either 4 or 12 weeks of age. All rats were bred in an established colony at the University of Alberta and were maintained in a controlled environment as described in Chapter 2.

MEDICA 16 Treatment

In one series of experiments, JCR:LA-cp rats were treated for 6 weeks with 0.25% MEDICA 16 in food from age 6 weeks to 12 weeks. All animals were studied in the non-fasted state at the end of the dark phase.

Tissue and Blood Isolation

Animals were anesthetized as described in Chapter 2 and cardiac puncture was performed to obtain 3-6 ml blood samples (depending on the age and size of the animal). Because AMPK can be artificially activated if tissues are not isolated rapidly and freeze-clamped in liquid nitrogen, extreme care was taken in order to minimize the activation of AMPK. Liver and gastrocnemius muscle were harvested within 30 sec after cardiac puncture, frozen immediately in liquid N_2 and stored at -80°C until analysis. Visible adipose tissue was quickly dissected from the gastrocnemius prior to freezing.

Plasma Measurements

Samples were analyzed for plasma glucose, insulin, fatty acid, TG, and leptin levels as described in Chapter 2.

Tissue Extractions

Tissue TG levels were determined using silicic acid separation as described in Chapter 2. ACC activity was performed on 6% PEG fractions as described in Chapter 2. AMPK activity were performed on 6% PEG fractions using the SAMS peptide as described in Chapter 2.

Immunoblotting

6% PEG fractions were also used for immunoblotting with peroxidase-labeled streptavidin for determination of ACC protein expression as described in Chapter 2.

Statistical Analysis

All data are presented as the mean ± standard error (n). The data were analyzed with the statistical program Instat 2.01. ANOVA was used to evaluate the statistical significance of differences among groups. When necessary, data was log transformed in order to test for statistical significance. Two-tailed values of $p<0.05$ were considered significant.

Results

Insulin resistance in the JCR:LA-cp rat occurs between the ages of 4 and 12 weeks (25). Body weight and various plasma measurements for 4- and 12-week JCR:LA-cp and their lean age-matched controls is shown in Table 5-1. At 4-weeks of age there was no difference in body weight between the JCR:LA-cp and lean age-matched controls. At 12 weeks of age, both JCR:LA-cp and lean age-matched control rats had significantly higher body weights compared with 4-week rats. The JCR:LA-cp rats also had a significantly elevated body weight compared with the lean age-matched controls. Plasma insulin levels began to rise at 4-weeks of age in the JCR:LA-cp rats, with a profound hyperinsulinemia ensuing by 12 weeks of age. In contrast, plasma glucose levels were not significantly different in the JCR:LA-cp rats in either the 4-week or 12-week old rats, compared with lean age-matched controls.

Plasma fatty acid levels were not significantly different between JCR:LA-cp rats at 4-weeks of age, compared with lean age-matched controls. However, at 12 weeks of age fatty acid levels were significantly elevated in the JCR:LA-cp rats compared with age-matched controls. Fatty acid levels also increased slightly in the lean rats with age. Plasma TG levels were significantly elevated at 4-weeks of age in the JCR:LA-cp rats compared with lean age-matched controls. By 12 weeks, plasma TG levels were almost double that of the 4-week JCR:LA-cp rats, and were significantly higher than the 12-week lean age-matched controls. Plasma TG levels in the lean rats did not rise significantly with age.

At 4-weeks of age leptin levels in the JCR:LA-cp rats were elevated 13-fold compared with lean age-matched controls. Leptin levels continued to increase and were 30-fold greater than those of the lean age-matched controls at 12 weeks. Plasma leptin levels in the lean rats did not increase with age or body weight.

Fig. 5-1 shows that at 4-weeks of age, JCR:LA-cp rats have a 3-fold greater liver TG content than lean age-matched controls. There was no further increase in TG content in the liver of 12-week-old JCR:LA-cp rats compared with lean age-matched controls. The gastrocnemius of the 4-week JCR:LA-cp rats also contained a 3-fold greater amount of TG compared with the lean age-matched controls. However, by 12 weeks of age the TG content was more than 7-fold higher than the lean controls.

At 4-weeks of age, hepatic ACC activity measured in the presence of 10 mM citrate (Fig. 5-2) was already significantly elevated in the JCR:LA-cp rats compared with lean age-matched controls. ACC activity measured in the absence of citrate was not significantly different between the lean and JCR:LA-cp rats (1.6 ± 0.1 (n=6) vs. 1.4 ± 0.1 (n=6) nmol $\cdot$ min $^{-1}$ $\cdot$ mg protein $^{-1}$). The presence of 10 mM citrate resulted in a 1.5-fold increase in ACC activity compared with ACC activity measured in the absence of citrate in the 4-week lean age-matched controls, while the presence of 10 mM citrate resulted in a 3-fold increase in ACC activity compared with ACC activity measured in the absence of citrate in the 4-week JCR:LA-cp group.

A further increase was seen at 12 weeks in the JCR:LA-cp rats, with ACC activity measured in the presence of 10 mM citrate being 2-fold higher than 12-week lean age-matched controls. ACC activity measured in the absence of citrate was not significantly different between the lean and JCR:LA-cp rats (2.1 ± 0.2 (n=10) vs. 1.5 ± 0.1 (n=11) nmol · min $^{-1}$ · mg protein $^{-1}$). The presence of 10 mM citrate resulted in a 1.6-fold increase in ACC activity compared with ACC activity measured in the absence of citrate in the 12 week lean age-matched controls, while the presence of 10 mM citrate resulted in a 5.8-fold increase in ACC activity compared with ACC activity measured in the absence of citrate in the 12 week JCR:LA-cp group.

The increased ACC activity in the 12-week JCR:LA-cp rats was accompanied by a significant increase in ACC-265 protein expression compared with the 12-week lean age-matched controls. The increased ACC activity in the 4-week JCR:LA-cp rats was also accompanied by a slight increase in protein expression, although this increase did not reach statistical significance compared with the 4-week age-matched controls.

To determine if the increase in hepatic ACC in the JCR:LA-cp was due to alterations in AMPK control of ACC, we measured AMPK activity in 4 and 12 week old JCR:LA-cp and lean age-matched control rats (Fig. 5-3). No significant difference in maximally stimulatable AMPK activity was seen in the 4-week

JCR:LA-cp rats compared with the lean age-matched controls. AMPK activity measured in the absence of AMP was not significantly different between the 4-week JCR:LA-cp and lean age-matched control group (740 ± 48 (n=6) vs. 730 ± 69 (n=6) pmol $\cdot$ min $^{-1}$ $\cdot$ mg protein $^{-1}$). The presence of 200 μM AMP resulted in a 1.7-fold increase in AMPK activity compared with AMPK activity measured in the absence of AMP in both the 4-week lean and JCR:LA-cp groups.

However, maximally stimulatable hepatic AMPK activity in the 12-week JCR:LA-cp rats was increased compared with the 4-week JCR:LA-cp rats, and was significantly increased compared with the 12-week lean controls. AMPK activity in the absence of AMP was also significantly increased in the 12-week JCR:LA-cp rats compared with the lean age-matched controls (1220 ± 117 (n=11) vs.654 ± 40 (n=8) pmol $\cdot$ min $^{-1}$ $\cdot$ mg protein $^{-1}$). The presence of 200 μM AMP resulted in a 2-fold increase in AMPK activity compared with AMPK activity measured in the absence of AMP in both the 12-week lean and JCR:LA-cp groups.

Skeletal muscle has been implicated as an important site of insulin resistance (3), and TG accumulation within the muscle has been implicated as contributing to insulin-resistance (4). We therefore measured ACC activity and protein expression in gastrocnemius muscle (Fig. 5-4). However, no differences were seen in maximally stimulatable ACC activity between JCR:LA-cp and lean age-matched control rats, at either 4 or 12 weeks of age. There was no significant difference in ACC activity measured in the absence of citrate in the 4-week lean and JCR:LA-cp rats (0.17 ± 0.02 (n=6) vs. 0.17 ± 0.01 (n=6) nmol $\cdot$ min $^{-1}$ $\cdot$ mg protein $^{-1}$).

However, 10 mM citrate increased ACC activity 6.6-fold compared with ACC activity measured in the absence of citrate in the 4-week lean skeletal muscle, while 10 mM citrate increased ACC activity 9.5-fold compared with ACC activity measured in the absence of citrate in the 4-week JCR:LA-cp muscle. There was no significant difference in ACC activity measured in the absence of citrate in the 12-week lean and JCR:LA-cp rats (0.19 ± 0.05 (n=9) vs. 0.24 ± 0.06 (n=8) nmol $\cdot$ min $^{-1}$ $\cdot$ mg protein $^{-1}$). 10 mM citrate increased ACC activity 6.6-fold compared with ACC activity measured in the absence of citrate in the 12-week lean skeletal muscle, while 10 mM citrate increased ACC activity 11-fold compared with ACC activity measured in the absence of citrate in the 12-week JCR:LA-cp muscle.

Although a trend for a decreased expression of the ACC-280 isoform occurred in the 12-week JCR:LA-cp rats compared with the lean age-matched controls, this difference was not statistically significant. However, this decrease did result in the expression of the two isoforms being relatively equal in the JCR:LA-cp rats, rather than the 280 kDa isoform predominating. There was no difference in maximally stimulatable AMPK activity in the JCR:LA-cp rats or lean age-matched controls at either age (Figure 5-5). There was also no difference in AMPK activity measured in the absence of AMP between the 4-week JCR:LA-cp muscle and lean age-matched controls (210 ± 25 (n=5) vs. 259 ± 16 (n=6) pmol $\cdot$ min $^{-1}$ $\cdot$ mg protein $^{-1}$). 200 μM AMP increased AMPK activity 1.5-fold compared with AMPK activity measured in the absence of AMP in both the 4-week JCR:LA-cp and lean age-matched muscles. There was also no difference in AMPK activity measured in the absence of AMP between the 12-week JCR:LA-cp muscle and lean age-matched controls (154 ± 19 (n=7) vs. 203 ± 25 (n=9) pmol · min $^{-1}$ · mg protein $^{-1}$). 200 μM AMP increased AMPK activity 1.5-fold compared with AMPK activity measured in the absence of AMP in both the JCR:LA-cp and lean age-matched muscles.

MEDICA 16 (β,β'-tetramethyl hexadecanedioic acid) is a long-chain fatty acyl analogue developed as a hypolipidemic and antiobesity-antidiabetogenic compound (26). Previous studies have demonstrated that MEDICA 16 significantly reduces intracellular TG content in gastrocnemius muscle and this is accompanied by an improvement in insulin sensitivity (25). We therefore treated JCR:LA-cp rats with MEDICA 16 for a 6-week period. This treatment protocol did not significantly change body weight or plasma glucose levels (Table 5-2). The modest loss of body weight was associated with a slight decrease in adipose tissue mass and a non-significant decrease in food intake. Plasma insulin levels did decrease, although they remained higher than values seen in the lean age-matched controls (Table 5-1).

As shown in Fig. 5-6, MEDICA 16 treatment significantly decreased maximally stimulatable liver ACC activity to levels seen in the lean age-matched controls. There was no significant difference in ACC activity measured in the absence of citrate in the MEDICA 16 treated compared with JCR:LA-cp rats (2.8 ± 0.7 (n=5) vs. 3.6 ± 0.9 (n=6) nmol · min $^{-1}$ · mg protein $^{-1}$). 10 mM citrate increased ACC activity 1.2-fold compared with ACC activity measured in the absence of citrate in the MEDICA 16 treated group, while 10 mM citrate increased ACC activity 2.3-fold compared with ACC activity measured in the absence of citrate in the 12-week JCR:LA-cp untreated group.

Interestingly, maximally stimulatable hepatic AMPK activity also decreased with MEDICA 16 treatment. Hepatic AMPK activity measured in the absence of AMP was also significantly reduced by MEDICA 16 treatment compared with JCR:LA-cp rats (303 ± 70 (n=5) vs. 2157 ± 300 (n=6) pmol $\cdot$ min $^{-1}$ $\cdot$ mg protein $^{-1}$). 200 μM AMP increased AMPK activity 2.8-fold compared with AMPK activity measured in the absence of AMP in both the MEDICA 16 treated group and JCR:LA-cp group.

In skeletal muscle, there was no significant change in ACC activity or AMPK activity (Fig. 6-7). However, we have observed that MEDICA 16 does not readily get into muscle tissue (Bar-Tana, unpublished results), which may explain the lack of effect in muscle.

TABLE 5-1.

Body Weight and Plasma Measurements of 4 and 12 week lean and JCR:LA-cp rats

	4-week		12 week	
	Lean (n=6)	JCR:LA-cp (n=6)	Lean (n=4)	JCR:LA-cp (n=8)
Body weight (grams)	87 ± 5	78 ± 4	324 ± 6†	399 ± 11*†
Insulin (pmol/l)	58 ± 5	431 ± 43*	272 ± 27†	2746 ± 439*†
Glucose (mmol/l)	8.6 ± 0.2	8.5 ± 0.2	9.5 ± 0.4	10.1 ± 0.7
FFA (mmol/l)	0.18 ± 0.01	0.16 ± 0.01	0.27 ± 0.02†	0.37 ± 0.06*†
Triglyceride (mmol/l)	1.4 ± 0.1	3.2 ± 0.1*	1.8 ± 0.2	6.4 ± 0.5*†
Leptin (ng/ml)	1.3 ± 0.4	16.5 ± 0.8*	1.2 ± 0.3†	36.9 ± 1.8*†

Values are means ± SE (n).

* Significantly different from lean age-matched controls.

† Significantly different from 4-week genotype matched.

TABLE 5-2.

Body Weight and Plasma Measurements of 12 week JCR:LA-cp and MEDICA 16-treated JCR:LA-cp rats

	JCR:LA-cp (n=4)	Medica 16 (n=8)
Body weight (grams)	465 ± 9	420 ± 10
Insulin (pmol/l)	2746 ± 439	1120 ± 161*
Glucose (mmol/l)	13.3 ± 1.0	12.4 ± 1.0

Values are means ± SE (n).

* Significantly different from control JCR:LA-cp rats.

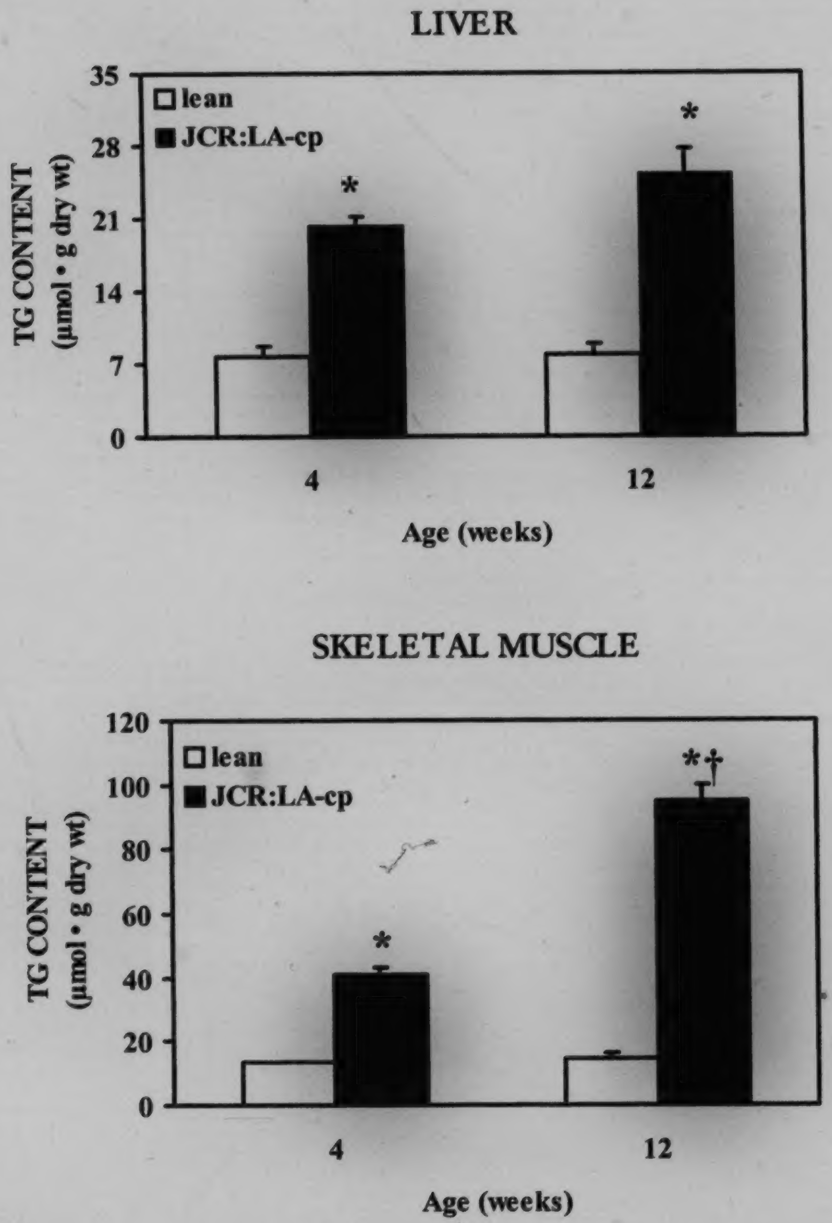
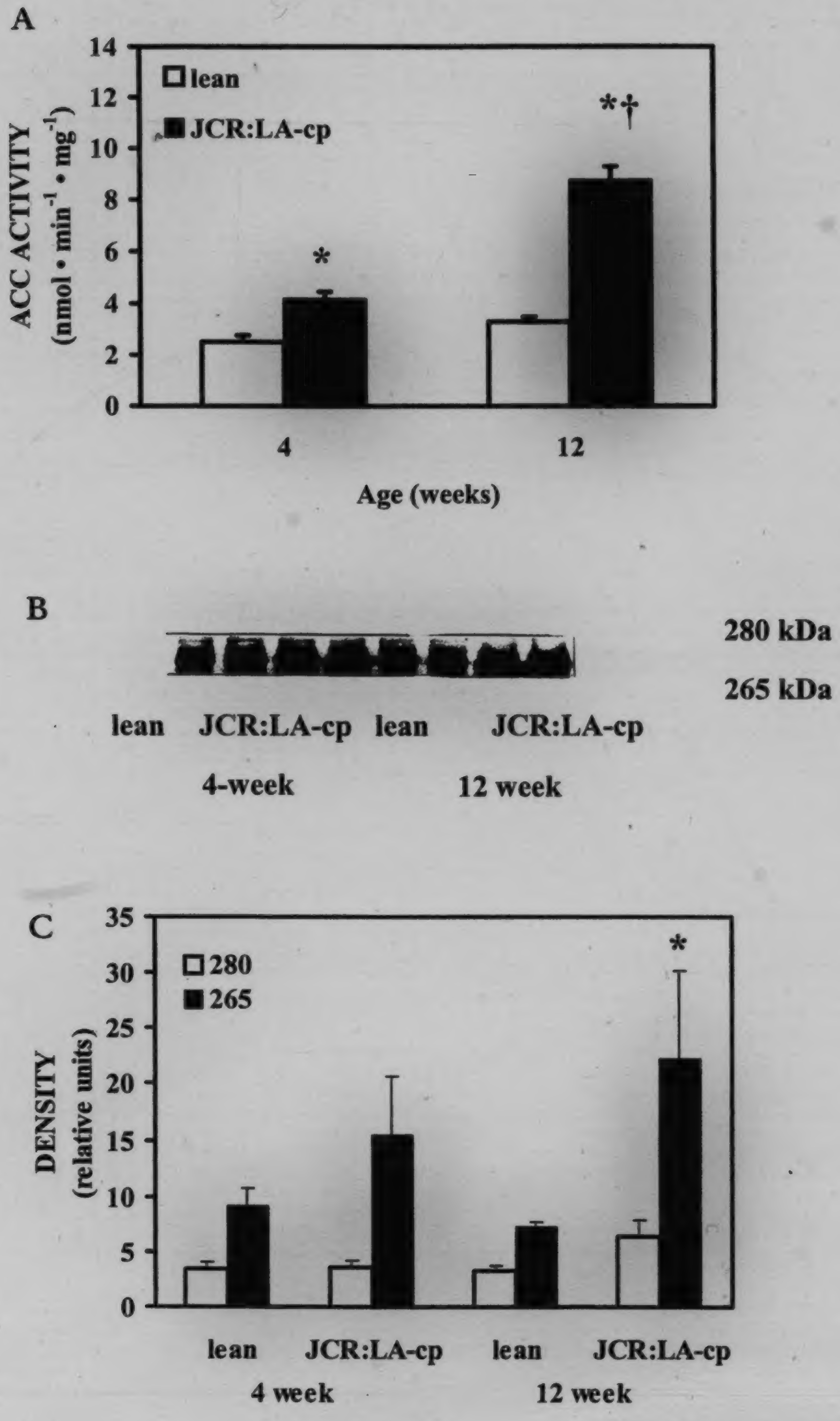


Figure 5-1. Triacylglycerol content of liver and skeletal muscle of JCR:LA-cp rats and lean age-matched controls. Values are mean ± SE of groups (n=6). *Significantly different from lean age-matched controls. †Significantly different from 4-week JCR:LA-cp rats.

Figure 5-2. Hepatic ACC activity (A) of JCR:LA-cp rats and lean age-matched controls. ACC activity was measured in the presence of 10 mM citrate. Protein expression of hepatic ACC-280 and ACC-265 isoforms (B) in JCR:LA-cp rats and lean age-matched controls. Rat heart was loaded in lane 1 to serve as a standard. Relative density of ACC isoforms (C) determined using a densitometry program. Values are mean ± SE (n=6 4-week lean, n=6 4-week JCR:LA-cp, n=10 12-week lean, n=11 12-week JCR:LA-cp). *Significantly different from lean age-matched controls. [†]Significantly different from 4-week JCR:LA-cp rats.


A
ACC ACTIVITY (nmol • min^{-1} • mg^{-1})
14
12
10
8
6
4
2
0
□ lean
■ JCR:LA-cp
*
*†
4
12
Age (weeks)
B
280 kDa
265 kDa
lean
JCR:LA-cp
lean
JCR:LA-cp
4-week
12 week
C
DENSITY (relative units)
35
30
25
20
15
10
5
0
□ 280
■ 265
*
lean
JCR:LA-cp
lean
JCR:LA-cp
4 week
12 week

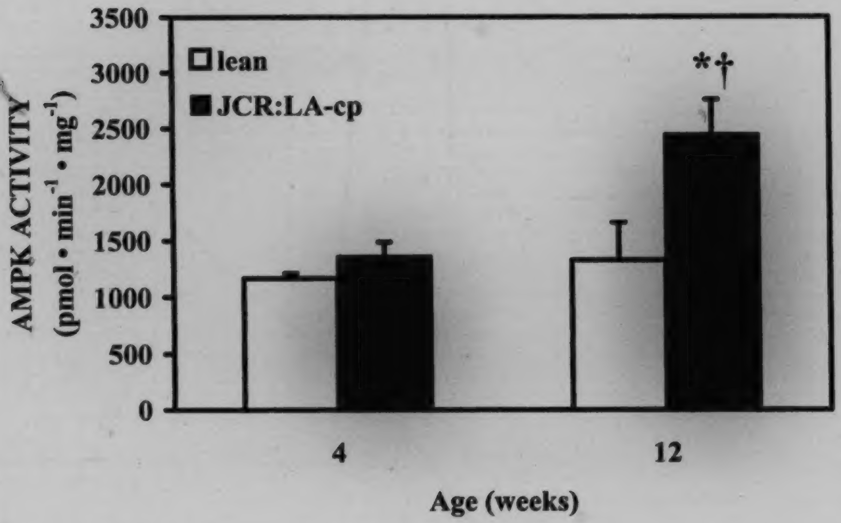
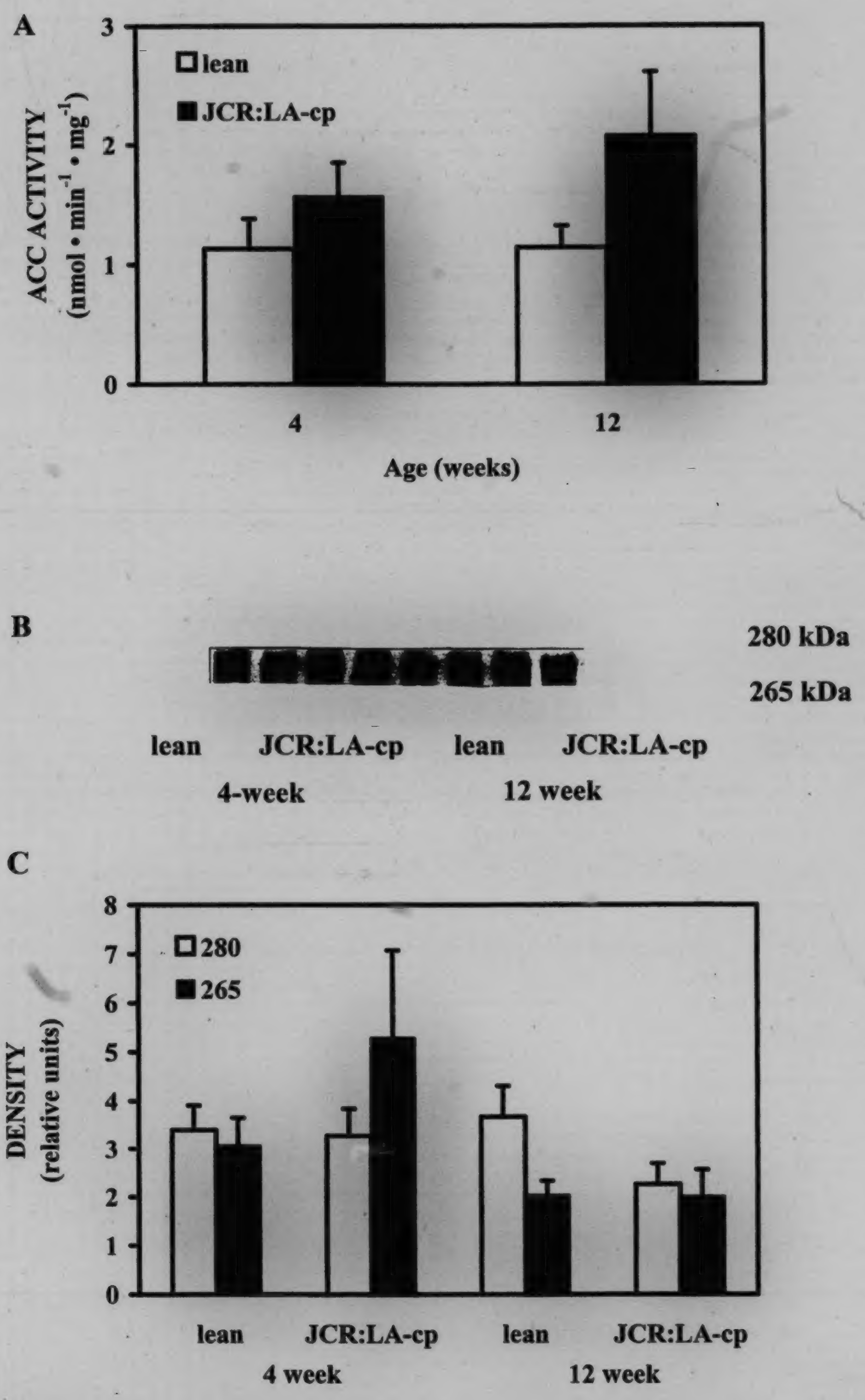


Figure 5-3. Hepatic AMPK activity measured in JCR:LA-cp rats and lean age-matched controls. AMPK activity was measured in the presence of 200 μM AMP. Values are mean ± SE (n=6 4-week lean, n=6 4-week JCR:LA-cp, n=8 12-week lean, n=11 12-week JCR:LA-cp). *Significantly different from lean age-matched controls. †Significantly different from 4-week JCR:LA-cp rats.

Figure 5-4. Skeletal muscle ACC activity (A) of JCR:LA-cp rats and lean age-matched controls. Protein expression of skeletal muscle ACC-280 and ACC-265 isoforms (B) in JCR:LA-cp rats and lean age-matched controls. Rat heart was loaded in lane 9 to serve as a standard. Relative density of ACC isoforms (C) determined using a densitometry program. Values are mean ± SE (n=6 4-week lean, n=6 4-week JCR:LA-cp, n=9 12-week lean, n=8 12-week JCR:LA-cp).


A
3
2
1
0
□ lean
■ JCR:LA-cp
ACC ACTIVITY
(nmol • min^{-1} • mg^{-1})
4
12
Age (weeks)
B
280 kDa
265 kDa
lean
JCR:LA-cp
lean
JCR:LA-cp
4-week
12 week
C
8
7
6
5
4
3
2
1
0
□ 280
■ 265
DENSITY
(relative units)
lean
JCR:LA-cp
lean
JCR:LA-cp
4 week
12 week

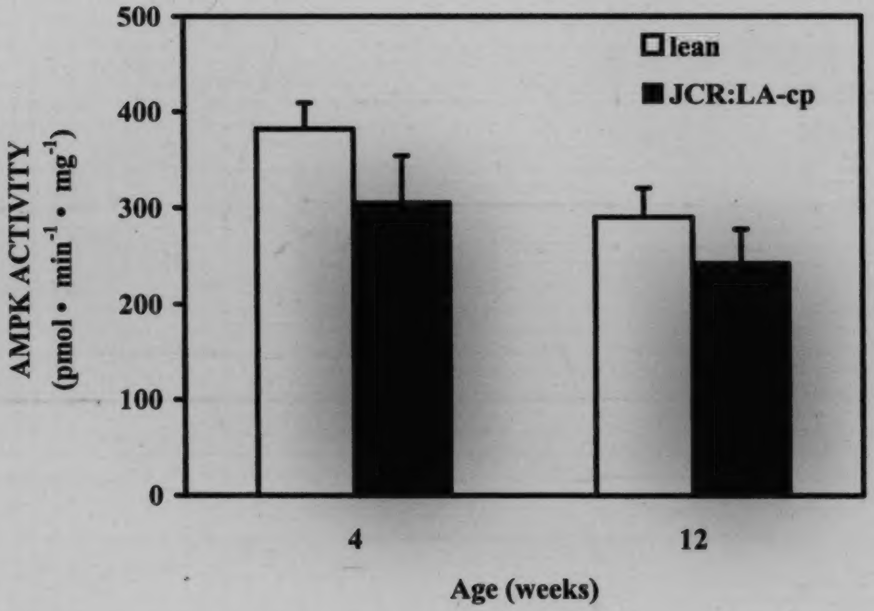


Figure 5-5. Skeletal muscle AMPK activity measured in JCR:LA-cp rats and lean age-matched controls. Values are mean ± SE of groups (n=6 4-week lean, n=6 4-week JCR:LA-cp, n=9 12-week lean, n=7 12-week JCR:LA-cp).

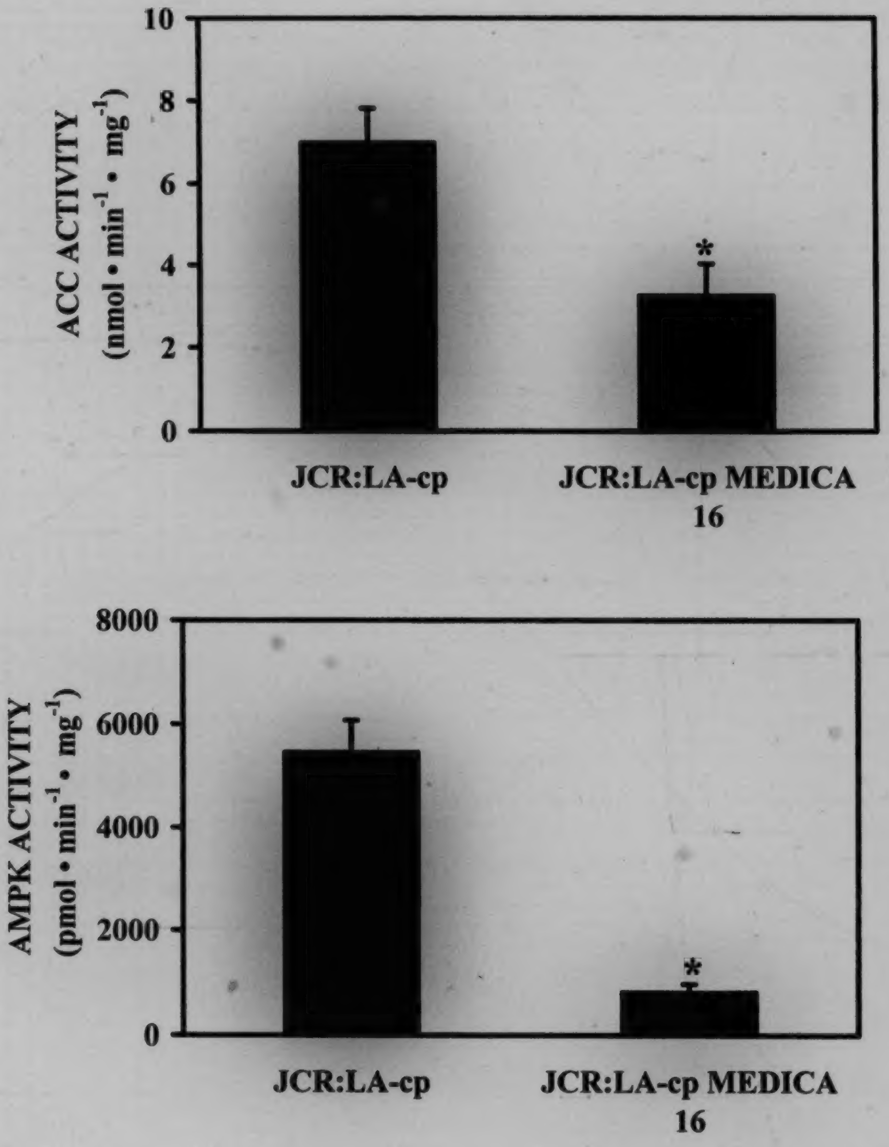


Figure 5-6. Effect of MEDICA 16 treatment on liver ACC and AMPK activity. Values are mean ± SE (n=6 JCR:LA-cp, n=5 MEDICA 16 treated). *Significantly different from untreated 12-week JCR:LA-cp rats.

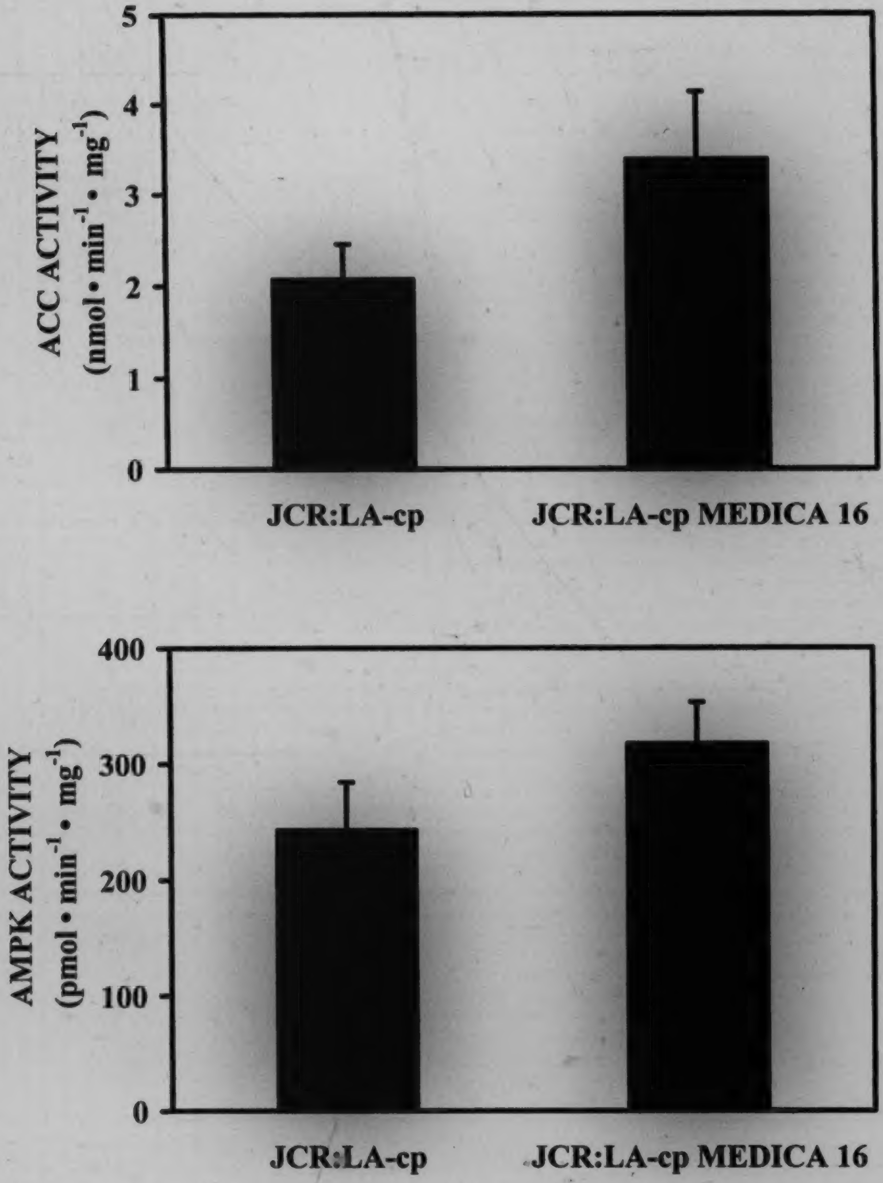


Figure 5-7. Effect of MEDICA 16 treatment on skeletal muscle ACC and AMPK activity. Values are mean ± SE (n=6 JCR:LA-cp, n=5 MEDICA 16 treated).

Discussion

At 4-weeks of age JCR:LA-cp rats have normal body weights, as well as normal plasma glucose and fatty acid levels. At this age, muscle glucose uptake in response to insulin is identical to lean controls (25). However, we demonstrate that increased levels of plasma insulin, TG and leptin are already apparent before the onset of muscle insulin resistance. By 12 weeks of age, the JCR:LA-cp rats displayed all the symptoms of insulin resistance, including obesity, hyperinsulinemia, and hyperleptinemia. In addition, plasma TG levels were markedly elevated in the JCR:LA-cp rats, as were both hepatic and skeletal muscle TG levels. In this study we demonstrate that increases in hepatic ACC activity and expression may be primarily responsible for the increase in TG content, whereas skeletal muscle ACC activity/expression did not correlate with the changes in TG content.

Several studies have claimed a link between TG content and the development of insulin resistance (1,2,3-6). In this study, we confirm that both the liver and muscle of insulin resistant rats contain elevated levels of TG. The measurement of intramuscular triglyceride is often artifactually elevated due to the presence of extramuscular adipose tissue (27). In order to address this issue, visible adipose tissue was quickly dissected from the gastrocnemius muscle prior to freezing. Furthermore, the presence of intracellular triglyceride in the gastrocnemius of JCR:LA-cp rats using electron microscopy and oil red O stain has been previously demonstrated (25). Thus although we can't completely rule the possibility that extracellular adipose tissue contributed to our measurements, our results are in agreement with several studies demonstrating a link between triglyceride accumulation and the development of insulin resistance.

The liver TG content of JCR:LA-cp rats was approximately 3-fold higher than the TG content in lean age-matched controls, at both 4 and 12 weeks of age. However, in gastrocnemius muscle, TG levels rose from 3-fold higher to 7-fold higher than lean age-matched controls between the ages of 4 and 12 weeks. This occurred despite the observation that at 4-weeks of age skeletal muscle from these rats is not insulin resistant as demonstrated by normal insulin-mediated glucose uptake (25). However, at 4-weeks of age, the JCR:LA-cp rats have elevated plasma insulin levels, indicative of whole body insulin resistance. This suggests that substantial accumulation of TG is an early event in the development of insulin resistance. It is tempting to speculate that there is a threshold level of TG accumulation within muscle prior to the development of insulin resistance. If so, this raises the possibility that continued accumulation of TG within the muscle may indeed play a causative role in the development of insulin resistance.

ACC not only catalyzes the rate-limiting step in fatty acid biosynthesis (9), it also has an important role in the regulation of muscle fatty acid oxidation, secondary to the formation of malonyl-CoA, a potent inhibitor of mitochondrial fatty acid uptake (10-12). In JCR:LA-cp rats hepatic ACC activity almost doubled by 4-weeks of age compared with lean age-matched controls, and increased even further by 12 weeks of age. This increase in ACC activity most likely contributes to the elevated plasma TG levels seen at 4-weeks of age, as well as the previously reported increase in plasma VLDL (28). The elevated ACC activity at 12 weeks was also accompanied by an increase in ACC-265 isoform expression, suggesting that increases in ACC activity and expression in the liver contribute to the abnormal plasma lipid levels and elevated hepatic TG content. The increase in ACC activity at 4-weeks was not accompanied by significant changes in protein expression. The reported increase in ACC activity was measured in the presence of 10 mM citrate, which represents maximally stimulated ACC activity. Under basal assay conditions (0 mM citrate) there was no change in ACC activity in JCR:LA-cp rats compared with lean controls (data not shown). Whether a decrease in phosphorylation was responsible for the increase in ACC activity was not addressed in this study.

We initially hypothesized that the increase in ACC activity in the liver was due to a decrease in AMPK activity in the JCR:LA-cp rats. However, the increase in hepatic ACC in the insulin resistant rats was associated with an increase in AMPK activity. Since AMPK and ACC can form a complex (29), the increase in AMPK activity may be a reflection of an increase in AMPK protein expression. However, this remains to be investigated. A recent study demonstrating that metformin activates hepatic AMPK may provide an alternative explanation (30). It is possible that the increase in hepatic AMPK is a compensatory mechanism to inhibit the increase in hepatic glucose production. This may provide an explanation for the lack of hyperglycemia in these animals.

A very large TG accumulation was seen within the gastrocnemius muscle of 12-week-old JCR:LA-cp rats (25). However, despite this we observed no significant changes in skeletal muscle ACC activity. Previous studies have demonstrated that obese rats and mice have increased muscle malonyl-CoA and triglyceride levels that are not associated with an increase in ACC activity (31). The increase in malonyl-CoA was attributed to an increase in cellular citrate levels (measured as citrate + malate), an allosteric activator of ACC (31). Citrate and malate levels were not determined in this study but may provide an explanation for the elevation in muscle TG content without changes in ACC activity. Furthermore, the enzymatic measurement of ACC activity cannot differentiate between the contributions of the different ACC isoforms. Thus, we also measured protein expression. Of interest was the observation that 12 week lean rats express almost twice the amount of the 280-kDa isoform compared with the 265-kDa isoform, while the JCR:LA-cp rats showed a decrease in 280-kDa expression such that the isoforms were expressed in almost a 1:1 ratio.

We also observed no changes in AMPK activity in the gastrocnemius of the JCR:LA-cp rats compared with the lean age-matched controls. AMPK activity was determined in a fed state at the end of the dark phase. However, these measurements do not indicate changes in AMPK activity that may occur throughout the day. While we are not aware of studies demonstrating a change in muscle AMPK activity in response to fasting and feeding, it has been demonstrated that hepatic AMPK is maximal during fasting and its activity is rapidly diminished during feeding (32). Because the time of sampling may influence AMPK activity, all samples were taken at the same time in a fed state. Furthermore, potential changes in AMPK expression or contraction-induced AMPK activity require further investigation.

Given the important role of AMPK in regulating glucose uptake and fatty acid oxidation in muscle [reviewed in (33)], we were surprised that no changes in AMPK activity were seen in insulin resistant muscle. AMPK has at least 2 isoforms of the α catalytic subunit (α1 and α2). The α2 isoform represents the majority of AMPK activity in muscle (34). At present little is known about the specific roles of the isoforms and whether specific isoforms are responsible for the control of fatty acid oxidation and glucose uptake. In the insulin resistant muscle, glucose uptake is completely blunted (25). The absence of changes in AMPK activity may suggest a defect in the upstream signaling or possibly several signals affecting AMPK activity. Clearly further studies are required to address this important issue.

MEDICA 16 is an ATP:citrate lyase inhibitor and chronic treatment with MEDICA 16 has previously been shown to reduce plasma TG concentrations by 80% and blunt the development of insulin resistance in the JCR:LA-cp rat (25) as well as in the related Zucker rat (35). Several studies have also shown acute effects of MEDICA 16. Acute treatment with MEDICA 16 results in a significant decrease in plasma VLDL cholesterol and triglyceride (36) and a significant inhibition of hepatic lipogenesis (37). *In vitro,* MEDICA 16 inhibits ACC activity (38) and induces a decrease in mitochondrial proton motive force that is accompanied by an increase in cellular respiration (39). In this study we demonstrate that chronic treatment of JCR:LA-cp rats for 6 weeks with MEDICA 16 dramatically reduces liver ACC activity. This supports the concept that increased hepatic ACC activity contributes to the elevated plasma TG levels seen in the insulin resistant JCR:LA-cp rats. Further support comes from studies using hydroxycitrate, another inhibitor of ATP:citrate lyase. Hydroxycitrate treatment of Zucker rats was also shown to reduce elevated lipogenic rates and plasma TG levels (40). Skeletal muscle ACC activity was unaffected by MEDICA 16 treatment, which may be due to the inability of MEDICA 16 to get into muscle tissue (Bar-Tana, unpublished observation).

Interestingly, the decrease in ACC activity with MEDICA 16 treatment was accompanied by a dramatic reduction in hepatic AMPK activity. These data suggest a dissociation in the changes in ACC activity from changes in AMPK activity in JCR:LA-cp rats, but supports the concept of a parallel change in ACC and AMPK activity. Although it has been suggested that activation of the AMPK signaling cascade may be effective in correcting insulin resistance (33), our data suggest that hepatic AMPK is activated in insulin resistance. However, if hepatic AMPK activity is increased to reduce hepatic glucose production, the use of AMPK activators may be useful in type 2 diabetes, a situation where hyperglycemia exists. It is thus important to characterize changes in AMPK signaling in models of type 2 diabetes.

Summary

In summary, our data show that an increase in liver ACC activity appears to contribute to the elevated plasma TG levels and probably the increased liver and muscle TG accumulation that occurs during insulin resistance. We recognize that these studies provide correlative evidence rather than a cause-effect relationship with regards to an increase in hepatic ACC activity and TG content. However, other studies have demonstrated that high-fat feeding results in an increase in hepatic ACC activity that is correlated with an increase in TG content (41). Furthermore, the inhibition of hepatic ACC activity with a fatty acid analog is associated with a decrease in TG content and reduction in VLDL secretion (42). Of interest, is the observation that these changes in TG and ACC precede the development of insulin resistance, suggesting a possible causative role of ACC and TG accumulation in the development of muscle insulin resistance (decreased insulin-stimulated glucose uptake). We also demonstrate that changes in AMPK activity do not mediate this increase in ACC activity and TG accumulation. Finally, treatment with MEDICA 16, which decreases TG accumulation (25) and the symptoms of insulin resistance (25,35), results in a decrease in hepatic ACC activity. This supports the concept that increased ACC activity and increased TG accumulation has an important role in the development of insulin resistance.

References

---

1. Forouhi NG, Jenkinson G, Thomas EL, Mullick S, Mierisova S, Bhonsle U, McKeigue PM, Bell JD: Relation of triglyceride stores in skeletal muscle cells to central obesity and insulin sensitivity in European and South Asian men. *Diabetologia* 42:932-935, 1999

2. Koyama K, Chen G, Lee Y, Unger RH: Tissue triglycerides, insulin resistance, and insulin production: implications for hyperinsulinemia of obesity. *Am J Physiol* 273:E708-E713, 1997

3. Perseghin G, Scifo P, De Cobelli F, Pagliato E, Battezzati A, Arcelloni C, Vanzulli A, Testolin G, Pozza G, Del Mashie A, Lazy L: Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: A ^{1}H-^{13}C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes* 48:1600-1606, 1999

4. Szczepaniak LS, Babcock EE, Schick F, Dobbins RL, Garg A, Burns DK, McGarry JD, Stein DT: Measurement of intracellular triglyceride stores by ^{1}H spectroscopy: validation in vivo. *Am J Physiol* 276:E977-E989, 1999

5. Pan DA, Lillioja S, Kriketos AD, Milner MR, Baur LA, Bogardus C, Jenkins AB, Storlein LH: Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* 46:983-988, 1997

6. Sreenan S, Keck S, Fuller T, Cockburn B, Burant CF: Effects of troglitazone on substrate storage and utilization in insulin-resistant rats. *Am J Physiol* 276:E1119-E1129, 1999

7. Shimabukuro M, Koyama K, Chen G, Wang MY, Trieu F, Lee Y, Newgard CB, Unger RH: Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc Natl Acad Sci USA* 94:4637-4641, 1997

8. Munday MR, Hemingway CJ: The regulation of acetyl-CoA carboxylase – a potential target for the action of hypolipidemic agents. *Advan Enzyme Regul* 39:205-234, 1999

9. Goodridge AG: Regulation of fatty acid synthesis in isolated hepatocytes. Evidence for a physiological role for long chain fatty acyl coenzyme A and citrate. *J Biol Chem* 248(12):4318-4326, 1973

10. Saddik M, Gamble J, Witters LA, Lopaschuk GD: Acetyl-CoA carboxylase regulation of fatty acid oxidation in the heart. *J Biol Chem* 268:25836-25845, 1993

11. Awan MM, Saggerson DE: Malonyl-CoA metabolism in cardiac myocytes and its relevance to the control of fatty acid oxidation. *Biochem J* 295:61-65, 1993

12. Veerkamp JH, Van Moerkerk HT: The effect of malonyl-CoA on fatty acid oxidation in rat and liver mitochondria. *Biochim Biophys Acta* 710:252-255, 1982

13. Mills SE, Foster DW, McGarry JD: Interaction of malonyl-CoA and related compounds with mitochondria from different rat tissues. Relationship between ligand binding and inhibition of carnitine palmitoyltransferase-1. *Biochem J* 214:83-91, 1983

14. Hardie DG: Regulation of fatty acid synthesis via phosphorylation of acetyl-CoA carboxylase. *Prog Lipid Res* 28:117-146, 1989

15. Allred JB, Reilly KE: Short-term regulation of acetyl-CoA carboxylase in tissues of higher animals. *Prog Lipid Res* 35:371-385, 1997

16. Sim ATR, Hardie DG: The low activity of acetyl-CoA carboxylase in basal and glucagon-stimulated hepatocytes is due to phosphorylation by the AMP-activated protein kinase and not cyclic AMP-dependent protein kinase. *FEBS Lett* 233:294-298, 1988

17. Kudo N, Gillespie JG, Kung L, Witters LA, Schulz R, Clanachan AS, Lopaschuk GD: Characterization of 5' AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. *Biochim Biophys Acta* 1301:67-75, 1996

18. Winder WW, Wilson HA, Hardie DG, Rasmussen BB, Hutber CA, Call GB, Clayton RD, Conley LM, Yoon S, Zhou B: Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. *J Appl Physiol* 82:219-225, 1997

19. Hardie DG, Carling D: The AMP-activated protein kinase: fuel gauge of the mammalian cell? *Eur J Biochem* 246:259-273, 1997

20. Ponticos M, Lu QL, Morgan JE, Hardie DG, Partridge TA, Carling D: Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *EMBO J* 17:1688-1699, 1998

21. Witters LA, Kemp BE: Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase. *J Biol Chem* 267:2864-2867, 1992

22. Gamble J, Lopaschuk GD: Insulin inhibition of 5' adenosine monophosphate activated protein kinase in the heart results in activation of acetyl coenzyme A carboxylase and inhibition of fatty acid oxidation. *Metabolism* 46:1270-1274, 1997

23. Beauloye C, Marsin AS, Bertrand L, Krause U, Hardie DG, Vanoverschelde JL, Hue L: Insulin antagonizes AMP-activated protein kinase activation by ischemia or anoxia in rat hearts, without affecting total adenine nucleotides. *FEBS Lett* 505:348-352, 2001

24. Wu-Peng WS, Chua SC Jr, Okada N, Liu S-M, Nicolson M, Leibel RL: Phenotype of the obese Koletsky (f) rat due to Tyr763Stop mutation in the extracellular domain of the leptin receptor (Lepr). *Diabetes* 46:513-518, 1997

25. Russell JC, Shillabeer G, Bar-Tana J, Lau DCW, Richardson M, Wenzel LM, Graham SE, Dolphin PJ: Development of Insulin Resistance in the JCR:LA-cp Rat: Role of triacylglycerols and effects of Medica 16. *Diabetes* 47:770-778, 1998

26. Bar-Tana J, Ben-Shoshan S, Blum J, Migron Y, Hertz R, Pill J, Rose-Kahn G, Witte E-C: Synthesis and hypolipidemic and antidiabetogenic activities of β,β,β',β'-tetrasubstituted, long-chain dioic acids. *J Med Chem* 32:2072-2084, 1989

27. Guo Z, Mishra P, Macura S: Sampling the intramyocellular triglycerides from skeletal muscle. *J Lipid Res* 42:1041-1048, 2001

28. Vance JE, Russell JC: Hypersecretion of VLDL, but not HDL, by hepatocytes from the JCR:LA-corpulent rat. *J Lipid Res* 31:1491-1501, 1990

29. Dyck JR, Kudo N, Barr AJ, Davies SP, Hardie DG, Lopaschuk GD: Phosphorylation control of cardiac acetyl-CoA carboxylase by cAMP-dependent protein kinase and 5'-AMP activated protein kinase. *Eur J Biochem* 262:184-190, 1999

30. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE: Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108:1167-1174, 2001

31. Saha AK, Vavvas D, Kurowski TG, Apazidis A, Witters LA, Shafrir E, Ruderman NB: Malonyl-CoA regulation in skeletal muscle: its link to cell citrate and the glucose-fatty acid cycle. *Am J Physiol* 272(35):E641-E648, 1997

32. Witters LA, Gao G, Kemp BE, Quistorff B: Hepatic 5'-AMP-activated protein kinase: zonal distribution and relationship to acetyl-CoA carboxylase activity in varying nutritional states. *Arch Biochem Biophys* 308:413-419, 1994

33. Winder WW, Hardie DG: AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* 277:E1-E10, 1999

34. Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, Teh T, House CM, Fernandez CS, Cox T, Witters LA, Kemp BE: Mammalian AMP-activated protein kinase subfamily. *J Biol Chem* 271:611-614, 1996

35. Mayorek N, Kalderon B, Itach E, Bar-Tana J: Sensitization to insulin induced by beta, beta'-methyl-substituted hexadecanedioic acid (MEDICA 16) in obese Zucker rats in vivo. *Diabetes* 46:1958-1964, 1997

36. Frenkel B, Bishara-Shieban J, Bar-Tana J: The effect of β,β'-tetramethylhexadecanedioic acid (MEDICA 16) on plasma very-low-density lipoprotein metabolism in rats: role of apolipoprotein C-III. *Biochem J* 298:409-414, 1994

37. Bar-Tana J, Rose-Kahn G, Srebnik M: Inhibition of lipid synthesis by ββ'-tetramethyl-substituted, C_{14}-C_{22}, α,ω-dicarboxylic acids in the rat *in vivo*. *J Biol Chem* 260:8404-8410, 1985

38. Rose-Kahn G, Bar-Tana J: Inhibition of rat liver acetyl-CoA carboxylase by beta, beta'-tetramethyl-substituted hexadecanedioic acid (MEDICA 16). *Biochim Biophys Acta* 1042:259-264, 1990

39. Hermesh O, Kalderon B, Bar-Tana J: Mitochondria uncoupling by a long chain fatty acyl analogue. *J Biol Chem* 273:3937-3942, 1998

40. Sullivan AC, Triscari J, Spiegel JE: Metabolic regulation as a control for lipid disorders. II. Influence of (--)-hydroxycitrate on genetically and experimentally induced hypertriglyceridemia in the rat. *Am J Clin Nutr* 30:777-784, 1977

41. Portillo MP, Chavarri M, Duran D, Rodriquez VM, Macarulla MT: Differential effects of diets that provide different lipid sources on hepatic lipogenic activities in rats under ad libitum or restricted feeding. *Nutrition* 17:467-473, 2001

42. Asiedu DK, al-Shurbaji A, Rustan AC , Bjorkhem I, Berglund L, Berge RK: Hepatic fatty acid metabolism as a determinant of plasma and liver triacylglycerol levels. Studies on tetradecylthioacetic and tetradecylthiopropionic acids. *Eur J Biochem* 227:715-722, 1995

CHAPTER 6.

Chronic Exercise Improves Whole Body Insulin Sensitivity in Insulin Resistant Rats: Role of AMP-Activated Protein Kinase Activation in the Regulation of Cardiac Triacylglycerol Content and Glycolysis

---

A version of this chapter has been submitted for publication in *Diabetes*. Atkinson LL, Kelly SE, Kozak J, Russell JC, Lopaschuk GD.

My role in this work involved performing all the biochemical experiments (except those noted below) as well as writing the manuscript. Ray Kozak performed the heart perfusions. Sandra Kelly taught me the exercise protocol. Ken Strynadka performed the HPLC analysis of CoA esters.

CHAPTER 6.

Chronic Exercise Improves Whole Body Insulin Sensitivity in Insulin Resistant Rats: Role of AMP-activated Protein Kinase Activation in the Regulation of Cardiac Triacylglycerol Content and Glycolysis

Abstract

In insulin resistant muscle, fatty acid flux is directed toward triacylglycerol (TG) storage rather than fatty acid oxidation, resulting in TG accumulation. Although we have identified an association between cardiac TG accumulation and reduced cardiac glycolysis in the insulin resistant JCR:LA-cp rat, it is not clear whether TG accumulation in the heart plays a causative role in the development of insulin resistance. In skeletal muscle, AMPK regulates the flux of fatty acids into oxidative pathways rather than TG storage and the activation of AMPK appears to mediate exercise-induced improvements in insulin sensitivity. We therefore investigated whether chronic exercise-training activates AMPK in the heart and whether the exercise-induced activation of AMPK was associated with a reduction in cardiac TG content and a stimulation of cardiac glycolysis in insulin resistant JCR:LA-cp rats. At the end of a 4-week exercise protocol, plasma insulin levels

were significantly reduced (178 ± 14 (n=26) vs. 361 ± 32 (n=26) and 1172 ± 92 (n=28) pmol $\cdot$ l^{-1}) in the exercise-trained rats compared with the weight-paired and sedentary controls. Insulin sensitivity was also significantly improved in the exercise-trained rats compared with the weight-paired and sedentary controls. Exercise-training significantly activates cardiac AMPK activity (1756 ± 92 (n=6) vs. 1067 ± 57 (n=6) and 1038 ± 71 (n=6) pmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) accompanied by a significant increase in cardiac glycolysis (2176 ± 143 (n=6) vs. 1564 ± 120 (n=5) and 1261 ± 277 (n=4) µmol glucose $\cdot$ g dry^{-1} $\cdot$ min^{-1}) and a significant reduction in cardiac TG content (12 ± 2 (n=6) vs. 19 ± 2 (n=6) and 27 ± 3 (n=6) µmol $\cdot$ g dry wt^{-1}) compared with weight-paired and sedentary control groups, respectively. However, palmitate oxidation rates, ACC activity and malonyl-CoA levels were significantly increased in both the exercise-trained and weight-paired groups compared with sedentary controls. Since the development of insulin resistance in the JCR:LA-cp rat can be delayed by early intervention with lipid-lowering agents, we investigated whether the benefits of 4-week of exercise-training could be sustained following cessation of exercise and food restriction. The beneficial effects of exercise on insulin sensitivity were reversed following the cessation of exercise and food restriction, and cardiac AMPK activity, fatty acid oxidation and glycolysis returned to sedentary control levels. Thus, this study demonstrates that chronic exercise activates AMPK in the heart and that the exercise-induced activation of AMPK is associated with a reduction in TG accumulation and an increase in cardiac glycolytic rates in insulin resistant rat hearts. Furthermore, chronic exercise followed by the cessation of exercise training and food restriction reverses the beneficial effects of exercise on insulin sensitivity and cardiac metabolism.

Introduction

Insulin resistance is a pre-type 2 diabetic state characterized by obesity, hyperinsulinemia, hyperlipidemia and impaired glucose tolerance (1-3). Altered fatty acid and glucose metabolism play a key role in the development of insulin resistance, although the mechanisms involved are not completely resolved. While the accumulation of cardiac TG has been demonstrated in insulin resistant rodents [reviewed in 4-6], controversy exists as to whether the accumulation of TG results from alterations in fatty acid supply, fatty acid oxidation or a combination of both (7). We have identified that TG accumulation is accompanied by decreased glycolytic rates in the insulin resistant JCR:LA-cp rat heart (8). However, it is not clear whether TG accumulation in the heart plays a role in the development of insulin resistance.

Currently, there is a great deal of interest in AMP-activated protein kinase (AMPK) as a therapeutic target in the treatment of obesity, insulin resistance, and type 2 diabetes (9-11). AMPK plays a key role in the regulation of both fatty acid and glucose metabolism [reviewed in 12]. The AMPK cascade acts as a metabolic sensor or 'fuel gauge' in the mammalian cell (12). This role is fulfilled by the ability of AMPK to phosphorylate key target proteins that control flux through metabolic pathways (12). When activated, AMPK increases fatty acid oxidation by inhibiting acetyl-CoA carboxylase (ACC) and reducing malonyl-CoA levels (13,14). AMPK activation also decreases TG content by inhibiting glycerol-3-phosphate acyltransferase (GPAT), the rate-limiting step in TG synthesis (15). Thus, through the coordinated regulation of key enzymes in fatty acid metabolism, AMPK regulates the flux of fatty acids into oxidation rather than TG storage (15). AMPK also plays a key role in the regulation of glucose metabolism. In the heart, the activation of AMPK results in a stimulation of glucose uptake via GLUT4 translocation (16) as well as the activation of glycolysis (17).

Recently AMPK has been identified as a target of the insulin sensitizing agents, metformin, troglitazone and rosiglitazone (18-20). Furthermore, the activation of AMPK has been implicated as the mediator of the beneficial effects of exercise on muscle insulin sensitivity (21-23). Consistent with this role, treatment of insulin resistant rats with AICAR, a non-specific AMPK activator, results in an improvement in insulin action (24). AICAR treatment of type 2 diabetic rodents also results in a significant reduction in plasma glucose levels (25-29). However, both AICAR and exercise have non-specific effects. For instance, exercise is accompanied by weight loss that leads to a reduction in plasma glucose, insulin, and lipid levels (30). Since AMPK activity can also be regulated by glucose (31), insulin (32-34) and fatty acids (35), it is not clear whether exercise activates AMPK directly or whether the exercise-induced activation of AMPK is indirect, possibly due to weight loss-induced reductions in plasma glucose, insulin or fatty acids.

In this study, we investigated the effect of chronic exercise on insulin sensitivity and cardiac energy metabolism in the insulin resistant JCR:LA-cp rat. Specifically, we determined whether exercise, independent of weight loss, activates AMPK in the heart and whether the exercise-induced activation of cardiac AMPK was associated with a reduction in cardiac TG accumulation and a stimulation of cardiac glycolysis in the insulin resistant JCR:LA-cp heart.

The development of insulin resistance in the JCR:LA-cp rat occurs between the ages of 4 and 12 weeks and is strongly associated with altered fatty acid metabolism (36). Since studies have demonstrated that the development of insulin resistance can be delayed by early treatment with the lipid-lowering agent, MEDICA 16 (36), we investigated whether the beneficial metabolic effects of exercise-training could be sustained following cessation of exercise-training and food restriction.

Methods

Animals

The study was performed on insulin resistant (cp/cp) male rats beginning at the age of 4-weeks.

Three groups of insulin resistant JCR:LA-cp rats were used in this experiment. A sedentary control group was allowed free access to food and water. A weight-paired group was food-restricted to maintain identical body weight to the exercise-trained group. Animals in the weight-paired group were started one day later than the exercise-trained group. Therefore, it could be estimated how much food to give the weight-paired group in order to maintain identical body weights to the exercise-trained group. The exercise-trained group was mildly food restricted and allowed free access to the running wheel. It has been previously demonstrated that the obese JCR:LA-cp rat requires mild food restriction in order to start running (37). All animals were maintained in a 12:12h light-dark cycle with the light cycle beginning at 11 am. Daily recordings and measurements were taken at the end of the dark cycle.

During the 4-week experimental protocol, body weight, food intake and water intake were recorded daily. Plasma sampling was performed as described in Chapter 2, with fasted blood samples obtained from the animals prior to the start of the experiment, at 6.5 weeks and at 8.5 weeks of age. A meal tolerance test to determine insulin sensitivity was performed at 8.5 weeks of age.

During the 8 week experimental protocol, all groups were subjected to the 4-week experimental protocol as described above. However, at 8.5 weeks of age, exercise-training and weight-matching was stopped and all groups received free access to food and water for the remaining 4-weeks. Body weight, food intake and water intake was recorded daily. Fasted blood samples were obtained from the tail prior to the start of the experiment, at 6.5, 8.5, 10.5 and 12.5 weeks of age. A meal tolerance test to determine insulin sensitivity was performed at 12.5 weeks of age.

Exercise-trained and weight-paired JCR:LA-cp rats were housed in Wahman running wheels as described in Chapter 2.

Tissue Collection

Adult male rats were fasted overnight and anesthetized without restraint in a large glass jar with 3% halothane at 1 l/min O_2. In one series of experiments, hearts were rapidly isolated, frozen immediately in liquid N_2 and stored at $-80^{o}C$ for biochemical analysis. Visible adipose tissue was quickly dissected from the heart prior to freezing. In another series of experiments, hearts were subjected to a 40-minute aerobic perfusion for quantitation of glucose and fatty acid metabolism.

Plasma Measurements

Samples were analyzed for plasma glucose, insulin, free fatty acids and triacylglycerol levels as described in Chapter 2. A meal tolerance test was performed to assess whole body insulin sensitivity as described in Chapter 2.

Heart Perfusions

Isolated working hearts from insulin resistant JCR:LA-cp rats were subjected to an aerobic perfusion with a modified Krebs-Henseleit solution containing 0.8 mM palmitate, 5 mM glucose, 3% bovine serum albumin, 2.5 mM free Ca^{2+}, and 500 µU/ml insulin. Spontaneously beating hearts were perfused at an 11.5 mm Hg left atrial preload and an 80 mm Hg aortic afterload. Functional parameters were measured as described in Chapter 2

The hearts used for measurement of fatty acid oxidation and glycolysis rates were subjected to a 40-minute perfusion in perfusate containing [1-^{14}C]palmitate and D-[5-^{3}H]glucose. Palmitate oxidation and glycolytic rates were determined simultaneously by quantitative collection of $^{14}CO_2$ and $^{3}H_2O$ produced by the hearts. Steady-state rates of fatty acid oxidation and glycolysis were determined as described in Chapter 2. At the end of the perfusions, heart ventricles were quickly frozen with Wollenberger clamps cooled to the temperature of liquid N_2.

Tissue Extractions

TG was extracted from frozen tissue according to the method of Folch, as described in Chapter 2. CoA esters were extracted and subjected to HPLC analysis as described in Chapter 2. ACC activity was determined as described in Chapter 2. AMPK activity was determined using the AMARA peptide as described in Chapter 2.

Statistical Analysis

All data are presented as the mean ± standard error. The data were analyzed with the statistical program Instat 2.01®. One-way ANOVA and Neumman-Keuls post-hoc tests was used to determine the statistical significance of differences between the insulin resistant JCR:LA-cp exercise-trained, weight-paired and sedentary control groups. Two-tailed values of $p<0.05$ were considered significant.

Results

The JCR:LA-cp rat develops insulin resistance between the age of 4 and 12 weeks (36). Figure 6-1A depicts the experimental protocol used in the first part of this study. Three groups of insulin resistant JCR:LA-cp rats were used in this experiment: sedentary controls (no intervention), weight-paired (food restricted to maintain identical body weight to exercise-trained) and exercise-trained (4-weeks of voluntary wheel running). Animals were randomized into their respective experimental group at 4-weeks of age. The experimental protocol began at 4.5 weeks of age, after a baseline fasting blood sample was obtained. At the completion of the 4-week experimental protocol, a meal tolerance test was performed to determine potential changes in insulin sensitivity.

During the 8 week experimental protocol (Fig. 6-1B), all groups were subjected to the experimental protocol described above. At 8.5 weeks of age, after a fasting blood sample was obtained, exercise-training and weight-matching was stopped and all groups received free access to food and water for the remaining 4-weeks. A meal tolerance test was performed at 12.5 weeks of age.

Figure 2 demonstrates that the exercise-trained rats linearly increased their running over the first 2 weeks of the experimental protocol. Running distance during the last 2 weeks remained relatively stable, with the exercise-trained rats running on average 6.6 km per day.

At the beginning of the experimental protocol, body weight and food intake was similar among all groups. After the 4-week experimental protocol, body weight (Fig. 6-3A) and food intake (Fig. 6-3B) were reduced in the weight-paired and exercise-trained rats compared with the sedentary control group. Food intake in the weight-paired group was slightly less than the exercise trained group because the exercise group could eat more food than the weight-paired group and gain less weight due to the caloric cost of wheel running. Water intake was consistently matched with food intake in all groups (data not shown).

Table 6-1 shows the fasting plasma fatty acid and TG levels after the 4-week experimental protocol. Plasma fatty acid and TG levels were significantly reduced in the weight-paired and exercise-trained compared with the sedentary controls. However, exercise-training had no significant independent effect on plasma fatty acid and TG levels compared with the weight-paired controls. Plasma glucose levels were slightly but non-significantly reduced in the weight-paired and exercise-trained compared with sedentary controls (data not shown).

Fasting plasma insulin levels were significantly decreased in the weight-paired and exercise-trained compared with sedentary controls (Fig. 6-4). There was also a significant decrease in insulin levels in the exercise-trained rats compared with weight-paired group. To assess more accurately whole body insulin sensitivity at the end of the 4-week protocol, a meal tolerance test was performed. The meal tolerance test demonstrates the insulin response to a 5 gram test meal. The weight-paired and exercise-trained groups had significantly improved insulin sensitivity compared with the sedentary controls (Fig. 6-5A). There was also a significant improvement in insulin sensitivity in the exercise-trained compared with weight-paired group (Fig. 6-5B).

Cardiac function and metabolism was determined using isolated working hearts from the insulin resistant JCR:LA-cp rats hearts. Hearts were obtained from fasted rats immediately following the last bout of wheel running. Mechanical function was monitored continuously over the 40-minute perfusion period in the spontaneously beating isolated working hearts. All hearts were subject to a constant left atrial preload of 11.5 mmHg and an aortic afterload of 80 mmHg. As shown in Table 6-2, there was no significant difference in heart rate, peak systolic pressure, or cardiac output. Both coronary and aortic flows were also continuously monitored which allowed for the determination of cardiac work (the product of cardiac output and peak systolic pressure) as an index of mechanical function. There was no significant difference in cardiac work (Table 6-2) among the groups.

AMPK plays a key role in the regulation of cardiac fatty acid oxidation, TG synthesis and glucose metabolism (12-17). AMPK is activated by exercise in skeletal muscle and has been implicated as the mediator of exercise-induced benefits on glucose metabolism (21-23). Thus, we determined whether AMPK was activated in the heart after the 4-week experimental protocol. Figure 6-6A demonstrates that cardiac AMPK was significantly increased in the exercise-trained group compared with the weight-paired and sedentary controls. AMPK measured in the presence of 200 μM AMP was also significantly increased in the exercise-trained group compared with the weight-paired and sedentary controls (3549 ± 239 (n=6) vs. 2178 ± 290 (n=6) and 2135 ± 155 (n=6) pmol · min $^{-1}$ · mg protein $^{-1}$). 200 μM AMP increased AMPK activity 2-fold compared to AMPK activity in the absence of AMP in the exercise-trained, weight-paired and sedentary control groups.

We further investigated whether the activation of AMPK was accompanied by altered cardiac fatty acid and glucose metabolism in the insulin resistant JCR:LA-cp hearts. In isolated working rat hearts, there was a significant increase in glycolytic rates (Fig. 6-6B) and a significant reduction in TG content (Fig. 6-6C) in the exercise-trained rat hearts compared with the weight-paired and sedentary control rats. Both the exercise-trained and weight-paired hearts had a significant increase in palmitate oxidation rates (Fig. 6-7A). Similarly, ACC activity was decreased in both the exercise-trained and weight-paired hearts (Fig. 6-7B). ACC activity measured in the presence of 10 mM citrate was also significantly decreased in both the exercise-trained and weight-paired hearts compared with sedentary controls (5.0 ± 0.7 (n=6) and 4.7 ± 0.8 (n=6) vs. 8.3 ± 0.7 (n=6) nmol · min $^{-1}$ · mg protein $^{-1}$). 10 mM citrate increased ACC activity 2.3-fold compared to ACC activity measured in the absence of citrate in all groups. Malonyl-CoA levels (Fig. 6-7C) were similar in all groups.

Table 6-3 demonstrates that both the exercise-trained and weight-paired groups have an increased heart weight to body weight ratio compared with sedentary controls due to substantially reduced body weight. However, the exercise-trained hearts, in which AMPK is activated, had an increased heart weight compared with the weight-paired group resulting in a significant increase in heart weight to body weight ratio.

We further investigated whether the 4-week exercise or food restriction intervention could be sustained following 4-weeks of the cessation of exercise and food restriction. We also wanted to determine whether early intervention with exercise would improve insulin sensitivity at 12 weeks of age; the age when muscle insulin resistance is fully established.

By the age of 12 weeks, body weight, food intake and water intake were not significantly different between the weight-paired, exercise-trained and sedentary controls (Table 6-4). Similarly, plasma FFA and TG levels were no longer significantly different in the exercise-trained, weight-paired and sedentary control groups (Table 6-5). Fasting plasma insulin levels (Fig 6-8A) in the exercise-trained and weight-paired groups also returned to sedentary control levels following 4-weeks of cessation of exercise training and food restriction. The improved insulin sensitivity resulting from exercise-training and weight-pairing was also lost following the cessation of exercise training and food restriction (Fig. 6-8B).

In the exercise-trained hearts, cardiac AMPK activity, palmitate oxidation and glycolytic rates returned to sedentary control levels (Table 6-6). However, the exercise-trained hearts had a significant decrease in cardiac output at the age of

12.5 weeks (Table 6-7). Heart weight to body weight ratio was significantly increased in the exercise-trained and weight-paired groups compared with sedentary controls due to slightly reduced body weights (Table 6-7).

TABLE 6-1.

Fasting plasma fatty acid and TG levels

	Plasma Fatty Acid (mmol/l)		Plasma TG (mmol/l)	
	4.5 weeks	8.5 weeks	4.5 weeks	8.5 weeks
Sedentary Control	2.2 ± 0.2 (n=19)	2.7 ± 0.2 (n=29)	2.5 ± 0.3 (n=19)	3.9 ± 0.5 (n=29)
Weight-Paired	2.6 ± 0.2 (n=20)	1.8 ± 0.2* (n=28)	2.0 ± 0.3 (n=20)	1.3 ± 0.1* (n=28)
Exercise-Trained	2.6 ± 0.2 (n=20)	2.0 ± 0.2* (n=27)	1.7 ± 0.1 (n=20)	1.3 ± 0.1* (n=26)

Values are means ± SE (n).

* Significantly different from Sedentary Control group.

TABLE 6-2.

Mechanical function of isolated working JCR:LA-cp rat hearts

	Sedentary Control (n=4)	Weight-Paired (n=5)	Exercise-Trained (n=6)
Heart Rate (beats · min $^{-1}$)	257 ± 4	246 ± 12	280 ± 15
Peak Systolic Pressure (mmHg)	98 ± 6	97 ± 3	94 ± 3
HR X PSP (beats · min $^{-1}$ · mmHg $^{-1}$ · 10 $^{-3}$)	25 ± 1	24 ± 2	27 ± 2
Cardiac Output (ml · min $^{-1}$)	34 ± 7	30 ± 2	33 ± 4
Cardiac Work (ml · mmHg · min $^{-1}$ · 10 $^{-2}$)	34 ± 9	29 ± 1	31 ± 4

Values are means ± SE (n). Hearts were perfused with Krebs-Henseleit solution containing 0.8 mM palmitate, 5 mM glucose, 3% albumin and 500 µU/ml (3000 pmol/l) insulin. Hearts were subjected to a 11.5 mmHg left atrial preload and 80 mmHg aortic afterload. HR X PSP = heart rate X peak systolic pressure.

TABLE 6-3.

Heart weight:Body weight Ratio

	Body weight (g) (n=6)	Heart weight (g) (n=6)	Heart wt/Body wt (mg/g) (n=6)
Sedentary Control	302 ± 6	1.03 ± 0.03	3.45 ± 0.12
Weight-Paired	175 ± 4	0.73 ± 0.02	4.19 ± 0.10*
Exercise-Trained	172 ± 4	0.80 ± 0.02	4.69 ± 0.17*†

Values are means ± SE (n).

* Significantly different from Sedentary Control group.

† Significantly different from Weight-Paired group.

TABLE 6-4.

Body Weight and Food Intake at 12.5 weeks of age following 8 week experimental protocol

	Body weight (g)	Food intake (g)
Sedentary Control	367 ± 31 (n=11)	29 ± 2 (n=11)
Weight-Paired	316 ± 7 (n=10)	35 ± 2 (n=10)
Exercise-Trained	306 ± 28 (n=10)	38 ± 2 (n=10)

Values are means ± SE (n).

TABLE 6-5.

Fasting Plasma Measurements at 12.5 weeks of age following 8 week experimental protocol

	Plasma Fatty Acid (mmol/l)	Plasma TG (mmol/l)
Sedentary Control	3.45 ± 0.30 (n=11)	4.56 ± 0.48 (n=9)
Weight-Paired	3.78 ± 0.47 (n=10)	3.81 ± 0.25 (n=10)
Exercise-Trained	3.19 ± 0.12 (n=10)	3.71 ± 0.34 (n=10)

Values are means ± SE (n).

TABLE 6-6.

Parameters of fatty acid and glucose metabolism at 12.5 weeks of age following 8 week experimental protocol

	Sedentary Control (n=5)	Weight-Paired (n=4)	Exercise-Trained (n=4)
AMPK activity (pmol · min $^{-1}$ · mg $^{-1}$)	405 ± 58	444 ± 98	407 ± 57
Glycolysis (nmol ^{3}H glucose · min $^{-1}$ · g dry wt $^{-1}$)	1530 ± 413	1845 ± 285	1077 ± 194
Palmitate oxidation (nmol ^{14}C palmitate · min $^{-1}$ · g dry wt $^{-1}$)	379 ± 22	400 ± 31	392 ± 33

Values are means ± SE (n).

TABLE 6-7.

Mechanical function and heart weight:body weight ratio of isolated working JCR:LA-cp rat hearts at 12.5 weeks of age following 8 week experimental protocol

	Sedentary Control (n=5)	Weight-Paired (n=4)	Exercise-Trained (n=4)
Heart Weight (grams)	1.23 ± 0.03	1.17 ± 0.05	1.20 ± 0.06
Body Weight (grams)	422 ± 14	360 ± 17	376 ± 15
Heart wt:Body wt ratio (mg/g)	2.93 ± 0.11	3.26 ± 0.14*	3.20 ± 0.13*
Heart Rate (beats $\cdot$ min^{-1})	276 ± 41	232 ± 11	216 ± 36
Peak Systolic Pressure (mmHg)	100 ± 5	95 ± 7	108 ± 9
HR X PSP (beats $\cdot$ min^{-1} $\cdot$ mmHg^{-1} $\cdot$ 10^{-3})	27 ± 2	22 ± 2	22 ± 3
Cardiac Output (ml $\cdot$ min^{-1})	47 ± 4	40 ± 3	30 ± 4*†
Cardiac Work (ml $\cdot$ mmHg $\cdot$ min^{-1} $\cdot$ 10^{-2})	47 ± 5	38 ± 5	32 ± 5

Values are means ± SE (n). Hearts were perfused with Krebs-Henseleit solution containing 0.8 mM palmitate, 5 mM glucose, 3% albumin and 500 μU/ml (3000 pmol/l) insulin. Hearts were subjected to a 11.5 mmHg left atrial preload and 80 mmHg aortic afterload. HR X PSP = heart rate X peak systolic pressure. * Significantly different from sedentary control group. † Significantly different from weight-paired group.

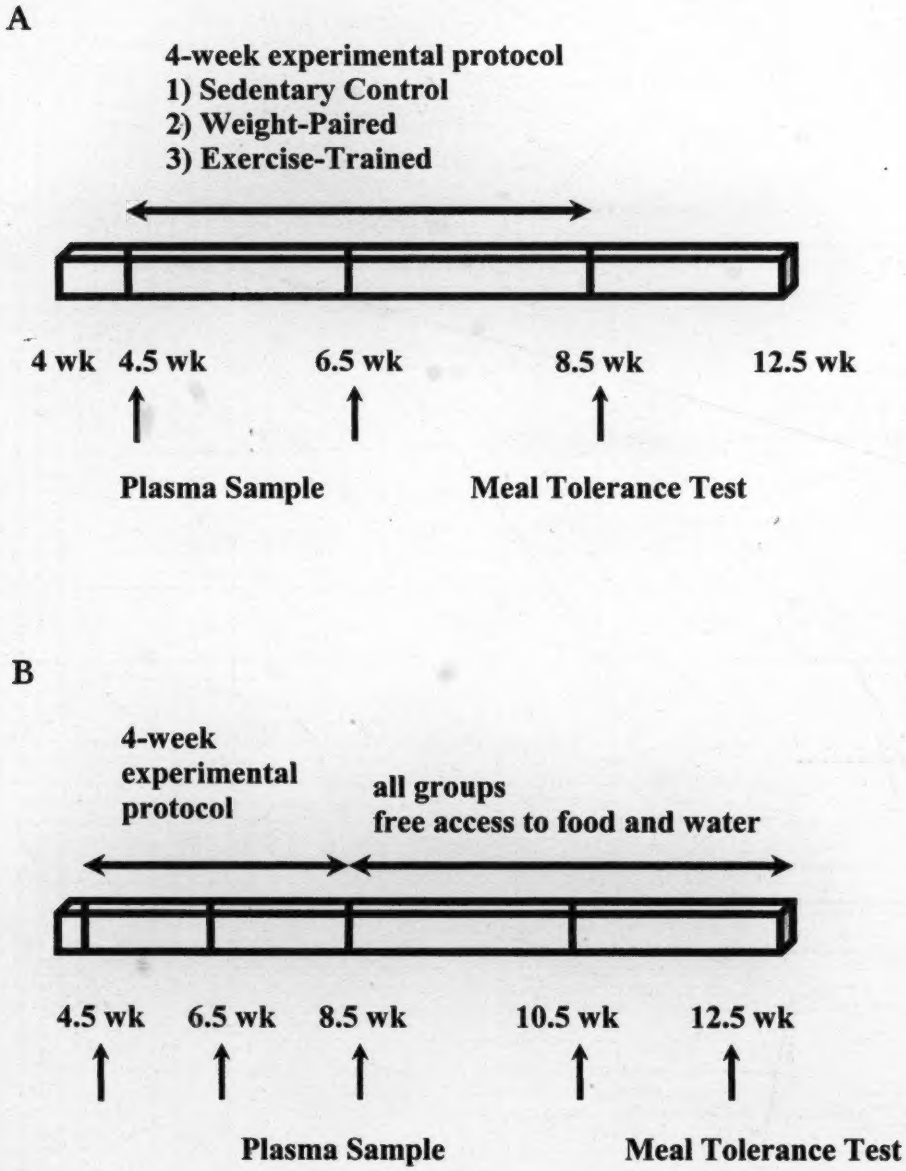

A
4-week experimental protocol
1) Sedentary Control
2) Weight-Paired
3) Exercise-Trained
4 wk
4.5 wk
6.5 wk
8.5 wk
12.5 wk
Plasma Sample
Meal Tolerance Test
B
4-week experimental protocol
all groups
free access to food and water
4.5 wk
6.5 wk
8.5 wk
10.5 wk
12.5 wk
Plasma Sample
Meal Tolerance Test

Figure 6-1. Scheme depicts 4 and 8 week experimental protocols. Insulin resistant JCR:LA-cp rats were randomized into experimental group at 4-weeks of age and 4-week experimental protocol began at 4.5 weeks of age.

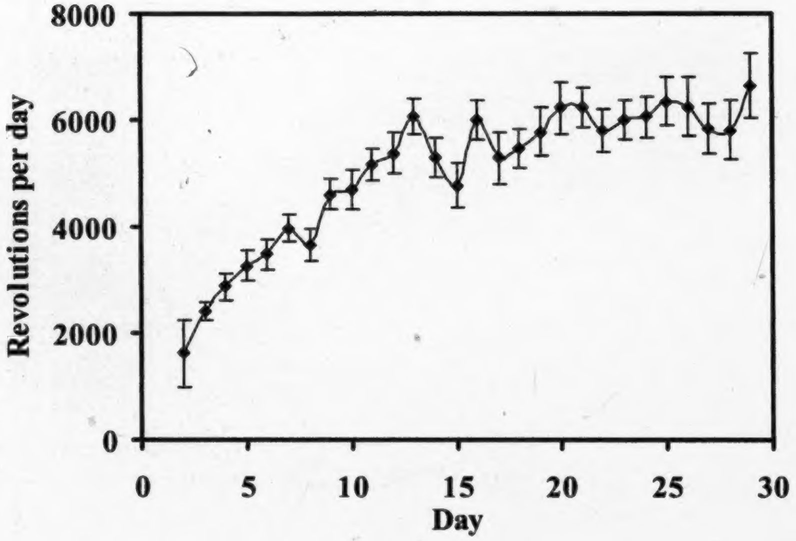


Figure 6-2. Wheel revolutions ran by the Exercise-Trained JCR:LA-cp rats. 1 revolution equals 1.1 meter. Values are means ± SE (n=25).

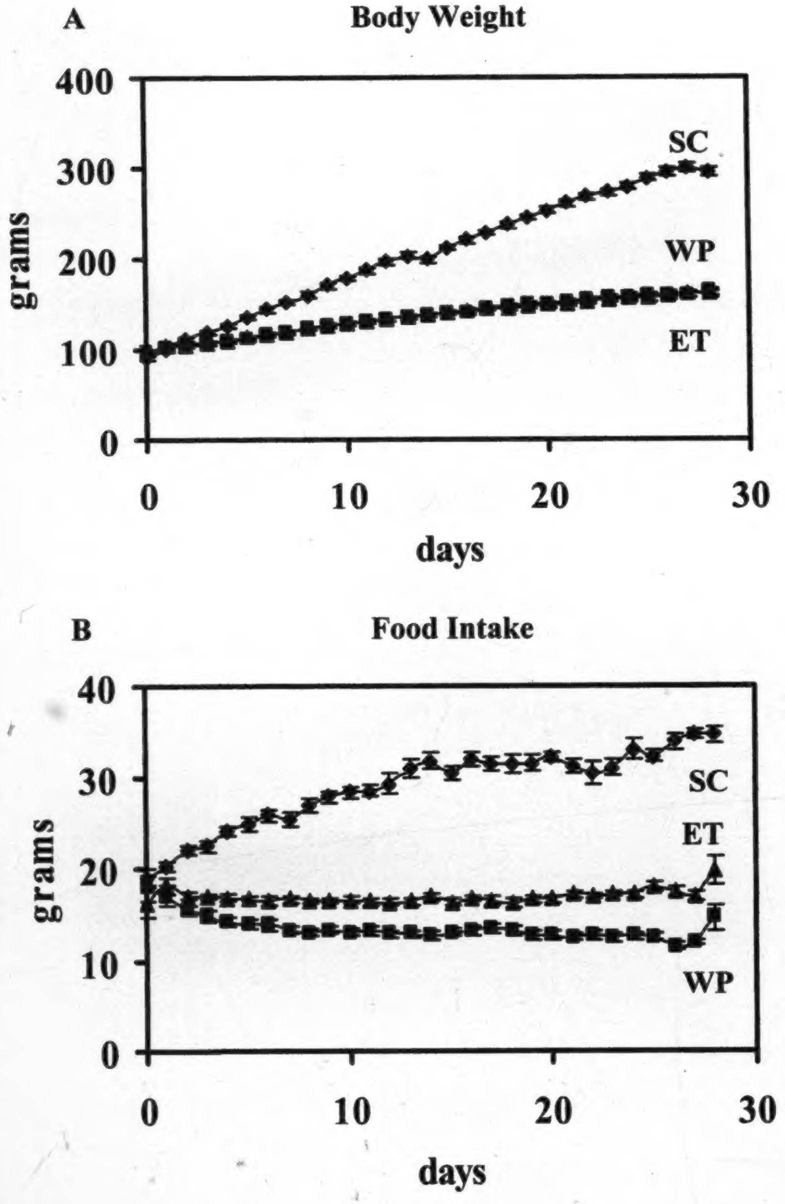


Figure 6-3. Body weight (A) and food intake (B) in Exercise-Trained (ET), Weight-Paired (WP) and Sedentary Control (SC) groups. Values are means ± SE (n=28 SC, n=26 WP, n=26 ET).

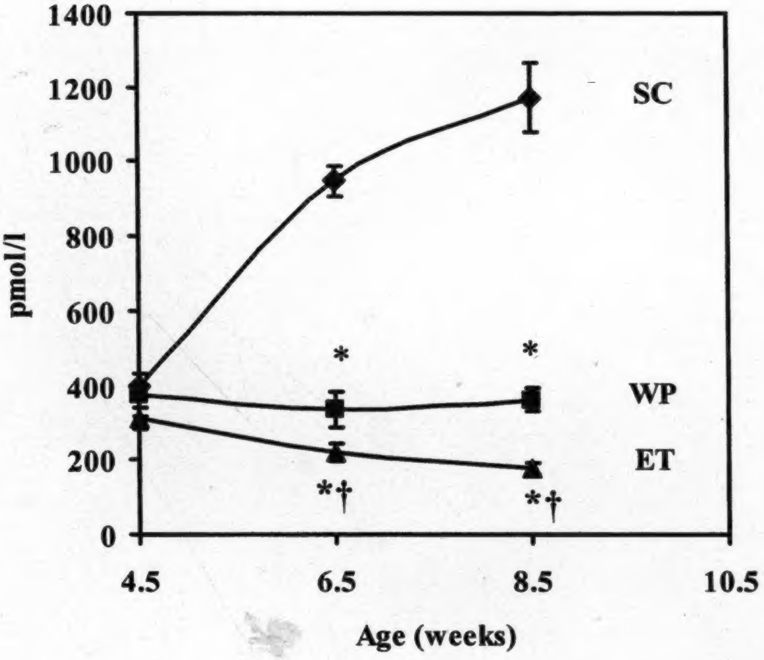


Figure 6-4. Fasting plasma insulin in the Exercise-Trained (ET), Weight-Paired (WP) and Sedentary Control (SC) groups. Values are means ± SE (n=28 SC, n=26 WP, n=26 ET). *Significantly different from Sedentary Control group. †Significantly different from Weight-Paired group.

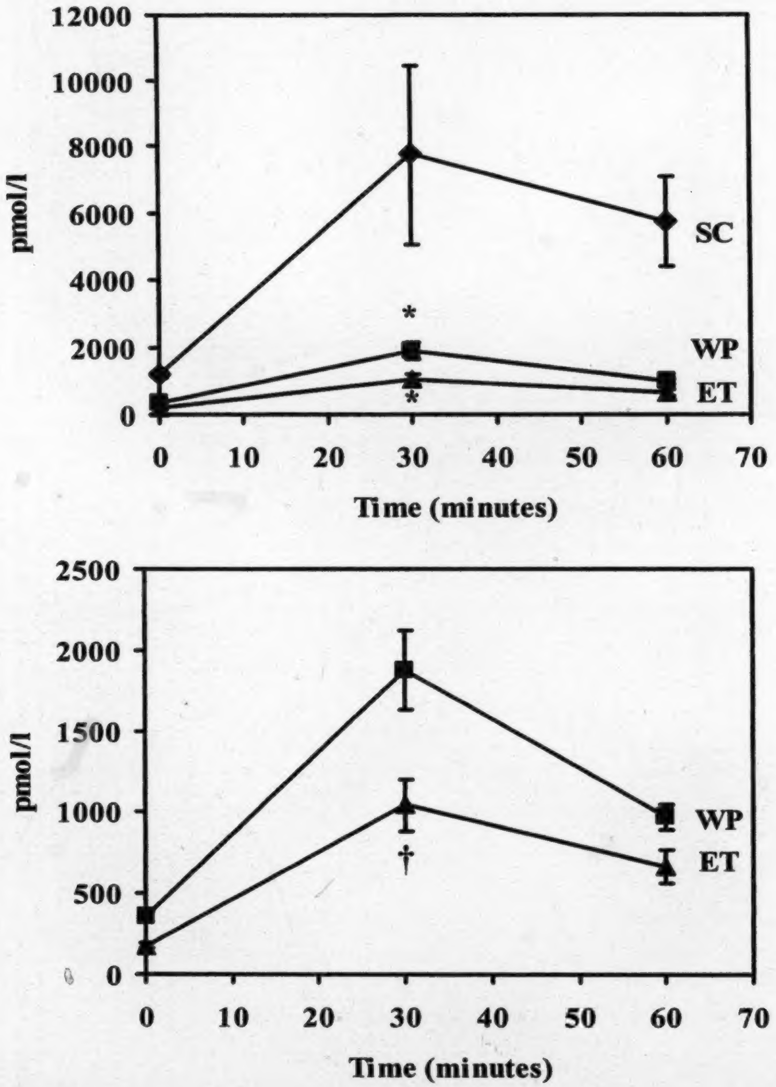
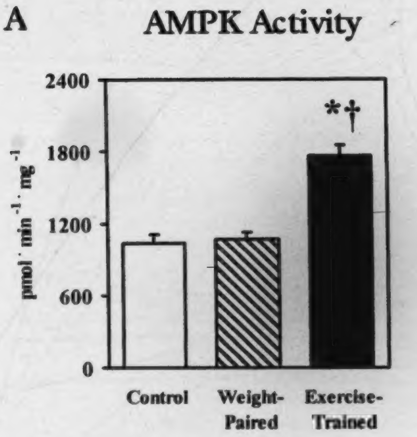
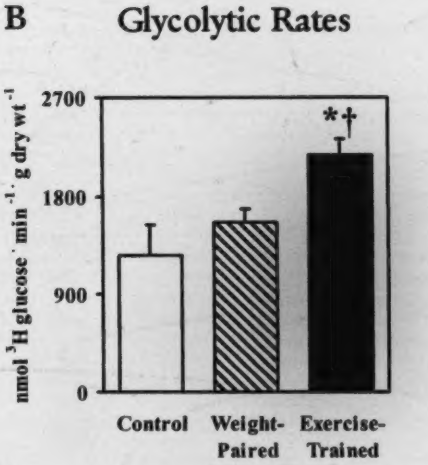


Figure 6-5. Insulin sensitivity in the Exercise-Trained (ET), Weight-Paired (WP) and Sedentary Control (SC) groups (A). Insulin sensitivity in the Exercise-Trained and Weight-Paired groups (B). Note scale difference on y axis. Values are means ± SE (n=29 SC, n=28 WP, n=26 ET). *Significantly different from Sedentary Control group. †Significantly different from Weight-Paired group.

Figure 6-6. Cardiac AMPK activity (A), glycolytic rates (B), and TG content (C) in Exercise-Trained, Weight-Paired and Sedentary Control groups. For AMPK activity and TG content, values are means ± SE (n=6/group). For glycolytic rates, values are means ± SE (n=4 SC, n=5 WP, n=6 ET). *Significantly different from Sedentary Control group. †Significantly different from Weight-Paired group.


A
AMPK Activity
pmol · min -1 · mg -1
2400
1800
1200
600
0
*†
Control
Weight-
Paired
Exercise-
Trained


B
Glycolytic Rates
nmol 3H glucose · min -1 · g dry wt -1
2700
1800
900
0
*†
Control
Weight-
Paired
Exercise-
Trained

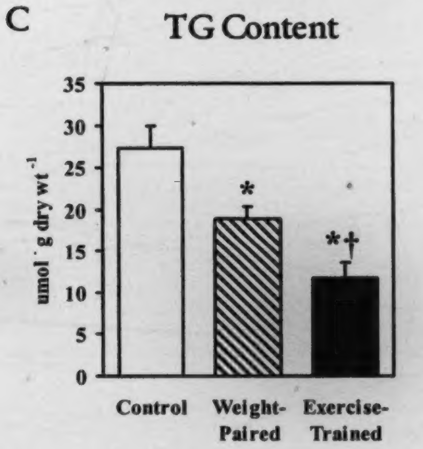
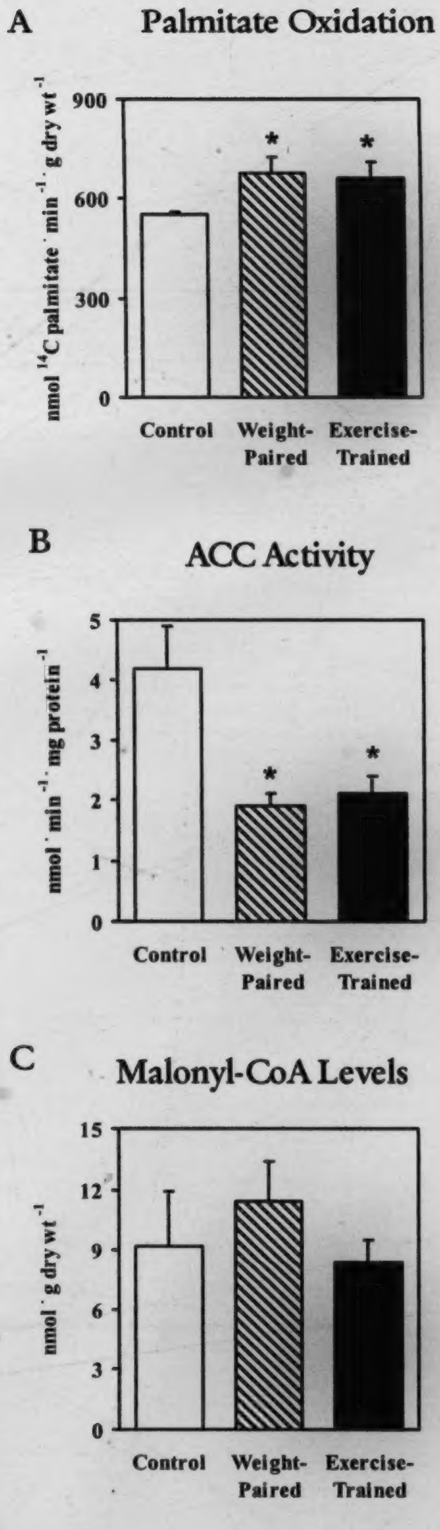

C
TG Content
umol · g dry wt -1
35
30
25
20
15
10
5
0
*
*†
Control
Weight-
Paired
Exercise-
Trained

Figure 6-7. Cardiac palmitate oxidation (A), ACC activity (B), and malonyl-CoA levels (C) in Exercise-Trained, Weight-Paired and Sedentary Control groups. For palmitate oxidation, values are means ± SE (n=4 SC, n=5 WP, n=6 ET). For ACC activity and malonyl-CoA, values are means ± SE (n=6/group). *Significantly different from Sedentary Control group.


A
Palmitate Oxidation
nmol ^{14}C palmitate · min^{-1} · g dry wt^{-1}
900
600
300
0
*
*
Control
Weight-Paired
Exercise-Trained
B
ACC Activity
nmol · min^{-1} · mg protein^{-1}
5
4
3
2
1
0
*
*
Control
Weight-Paired
Exercise-Trained
C
Malonyl-CoA Levels
nmol · g dry wt^{-1}
15
12
9
6
3
0
Control
Weight-Paired
Exercise-Trained

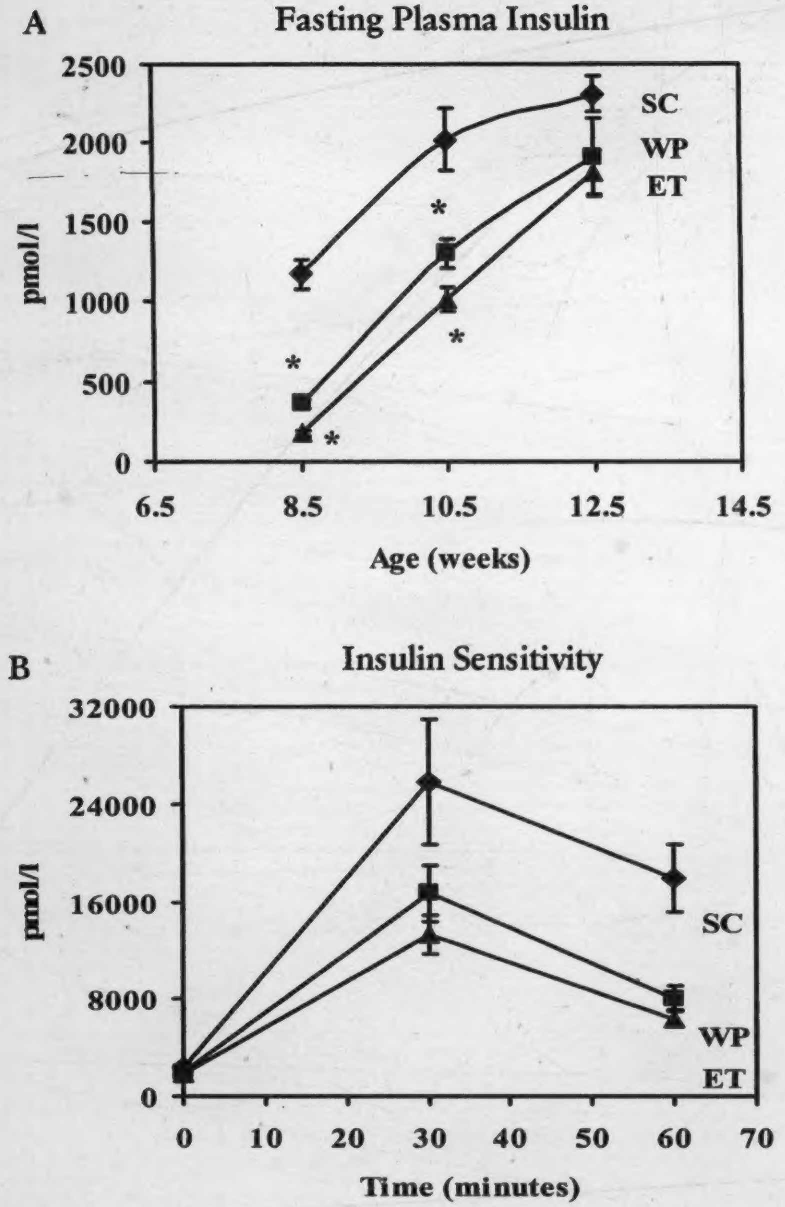


Figure 6-8. Fasting plasma insulin levels (A) and insulin sensitivity (B) in the Exercise-Trained (ET), Weight-Paired (WP) and Sedentary Control (SC) groups at 12.5 weeks of age following the 8 week experimental protocol. Values are means ± SE (n=11 SC, n=10 WP, n=6 ET). *Significantly different from Sedentary Control group.

Discussion

In this study, we demonstrate that exercise activates AMPK in the heart. The exercise-induced activation of AMPK was accompanied by a reduction in cardiac TG content and a stimulation of cardiac glycolysis. Furthermore, chronic exercise dramatically improved whole body insulin sensitivity and prevented the development of insulin resistance in the JCR:LA-cp rat. Following the cessation of exercise-training and food restriction, the improvement in insulin sensitivity was reversed and all parameters of cardiac metabolism returned to sedentary control levels.

Using isolated working hearts from insulin resistant JCR:LA-cp rats, we demonstrate that exercise-training significantly activates AMPK in the heart. Furthermore, the exercise-induced activation of AMPK was accompanied by a reduction in cardiac TG content and an elevation in cardiac glycolysis. We have previously demonstrated that the insulin resistant rat hearts have a 50% elevation in TG content associated with a 50% reduction in glycolytic rates compared with lean controls suggesting that TG accumulation may play a role in the development of insulin resistance in the heart (8). Consistent with this idea, the exercise-trained hearts had reduced cardiac TG content and increased rates of glycolysis that reverse to normal sedentary control levels following weight gain. Thus, we provide further evidence for a role of TG accumulation and reduced glucose utilization in the insulin resistant heart.

While the accumulation of cardiac TG has been demonstrated in insulin resistant rodents (7,8), controversy exists as to whether the accumulation of TG results from alterations in fatty acid supply, fatty acid oxidation or a combination of both. An increase in TG synthesis or a decrease in TG lipolysis may also contribute to TG accumulation. We previously demonstrated that fatty acid oxidation is unaltered in the isolated working insulin resistant JCR:LA-cp rat heart and suggested that increased fatty acid supply rather than alterations in fatty acid oxidation contribute to cardiac TG accumulation (8). Here, we demonstrate that both weight loss and exercise-training reduced plasma lipids, increased fatty acid oxidation and reduced cardiac TG content. Weight loss alone was unable to restore cardiac TG content to levels seen in lean control rats. However, in the exercise-trained hearts the activation of AMPK was accompanied by a normalization of TG content to lean control levels.

There is great interest in the AMPK signaling cascade as a novel therapeutic target for the treatment of obesity, insulin resistance and type 2 diabetes (22-24). The development of insulin resistance in the JCR:LA-cp rat occurs between the ages of 4 and 12 weeks and is strongly associated with elevated muscle TG (8,36). Furthermore, treatment with the lipid-lowering agent, MEDICA 16, during the development of insulin resistance, prevents the accumulation of muscle TG along with the development of insulin resistance (36). In this study, we demonstrate that exercise, during the critical time of insulin resistance development, prevents whole body insulin resistance. However, following the cessation of exercise-training and food restriction, beneficial effects of exercise were reversed.

The ability of weight loss and exercise to improve insulin sensitivity is well documented in both humans and rodents (30,39). In insulin resistant JCR:LA-cp rats, both chronic exercise-training and weight loss significantly reduced plasma insulin levels and improved insulin sensitivity. However, exercise-training reduced plasma insulin levels and improved insulin sensitivity to an even greater extent compared with weight loss alone. These data are consistent with human studies in which exercise-training and weight loss improve glucose tolerance and insulin sensitivity in an additive manner (30).

Exercise, in addition to improving insulin sensitivity, is well known to reduce the development of cardiovascular disease in humans (30,39) and has been shown to be cardioprotective in the JCR:LA-cp rat (40). We have previously demonstrated that the development of insulin resistance in the JCR:LA-cp rat is associated with an elevation in cardiac TG content and a reduction in cardiac glycolysis (8). Although TG accumulation has been associated with contractile dysfunction (7,41-45), we did not observe changes in cardiac function in the JCR:LA-cp rat heart. We have speculated that there may be a threshold level of TG accumulation required to cause contractile dysfunction (8), although, we have not yet investigated this possibility. However, previous data demonstrate that exercise-training can prevent the development of cardiovascular disease in the

JCR:LA-cp rat (40) suggesting that TG accumulation may contribute to cardiovascular disease in this model.

Summary

This study demonstrates that exercise significantly improves whole body insulin sensitivity and that the exercise-induced activation of AMPK in the heart is associated with a reduction in cardiac TG content and a stimulation of cardiac glycolysis. Furthermore, the cessation of exercise-training and food restriction reverses the exercise-induced improvements in insulin sensitivity and cardiac metabolism. However, the observation that AMPK activation in the heart is associated with an increase in heart weight to body weight ratio suggests that further studies are required to investigate the role of AMPK activation in the heart.

References

---

1. Reaven GM: Role of insulin resistance in human disease (syndrome X): an expanded definition. *Annu Rev Med* 44:121-131, 1993

2. Lebovitz HE: Insulin resistance: definition and consequences. *Exp Clin Endocrinol Diabetes* 109(Suppl 2):S135-S148, 2001

3. Alberti K, Zimmet P, Consultation W: Definition diagnosis and classification of diabetes mellitus and its complications. Part I: Diagnosis and classification of diabetes mellitus, provisional report of a WHO consultation. *Diabetes Med* 15:539-553, 1998

4. Kelley DE: Skeletal muscle triglycerides: an aspect of regional adiposity and insulin resistance. *Ann N Y Acad Sci* 967:135-145, 2002

5. Lewis GF, Carpentier A, Khosrow A, Giacca A: Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. *Endocrine Rev* 23:201-229, 2002

6. Unger RH: Lipotoxic diseases. *Annu Rev Med* 53:319-336, 2002

7. Young ME, Guthrie PH, Razeghi P, Leighton B, Abbasi S, Patil S, Youker KA, Taegtmeyer H: Impaired long-chain fatty acid oxidation and contractile dysfunction in the obese Zucker rat heart. *Diabetes* 51:2587-2595, 2002

8. Atkinson LL, Kozak R, Kelly SE, Onay-Besikci A, Russell JC, Lopaschuk GD: Potential mechanisms and consequences of cardiac triacylglycerol accumulation in insulin resistant rats. *Am J Physiol* [epub ahead of print] Dec 3, 2002

9. Moller DE: New drug targets for type 2 diabetes and the metabolic syndrome. *Nature* 414:821-827, 2001

10. Winder WW: AMP-activated protein kinase: possible target for treatment of type 2 diabetes. *Diabetes Technol Ther* 2:441-448, 2000

11. Winder WW, Hardie DG: AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* 277:E1-E10, 1999

12. Hardie DG, Carling D: The AMP-activated protein kinase: fuel gauge of the mammalian cell? *Eur J Biochem* 246:259-273, 1997

13. Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD: High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increae in 5' AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *J Biol Chem* 270:17513-17520, 1995

14. Kudo N, Gillespie JG, Kung L, Witters LA, Schulz R, Clanachan AS, Lopaschuk GD: Characterization of 5' AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. *Biochim Biophys Acta* 1301:67-75, 1996

15. Muoio DM, Seefeld K, Witters LA, Coleman RA: AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target. *Biochem J* 338:783-791, 1999

16. Russell RR 3rd, Bergeron R, Shulman GI, Young LH: Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol* 277:H643-H649, 1999

17. Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L: Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Curr Biol* 10:1247-1255, 2000

18. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE: Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108:1167-1174, 2001

19. Fryer LG, Parbu-Patel A, Carling D: The anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. *J Biol Chem* 277:25226-25232, 2002

20. Hawley SA, Gadalla AE, Olsen GS, Hardie DG: The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* 51:2420-2425, 2002

21. Tomas E, Zorzano A, Ruderman NB: Exercise and insulin signaling: a historical perspective. *J Appl Physiol* 93:765-772, 2002

22. Musi N, Fujii N, Hirschman MF, Ekberg I, Froberg S, Ljungqvist O, Thorell A, Goodyear LJ: AMP-activated protein kinase (AMPK) is activated in muscle of subjects with type 2 diabetes during exercise. *Diabetes* 50: 921-927, 2001

23. Stephens TJ, Chen ZP, Canny BJ, Michell BJ, Kemp BE, McConell GK: Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise. *Am J Physiol* 282: E688-E694, 2002

24. Iglesias MA, Ye JM, Frangioudakis G, Saha AK, Tomas E, Ruderman NB, Cooney GJ, Kraegen EW: AICAR administration causes an apparent enhancement of muscle and liver action in insulin-resistant high-fat fed rats. *Diabetes* 51:2886-2894, 2002

25. Bergeron R, Previs SF, Cline GW, Perret P, Russell RR 3rd, Young LH, Shulman GI: Effect of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside infusion on in vivo glucose and lipid metabolism in lean and obese Zucker rats. *Diabetes* 50:1076-1082, 2001

26. Fiedler M, Zierath JR, Selen G, Wallberg-Henriksson H, Liang Y, Sakariassen KS: 5-aminoimidazole-4-carboxy-amide-1-beta-D-ribofuranoside treatment ameliorates hyperglycaemia and hyperinsulinemia but not dyslipidemia in KKAy-CETP mice. *Diabetologia* 44:2180-2186, 2001

27. Song XM, Fiedler M, Galuska D, Ryder JW, Fernstrom M, Chibalin AV, Wallberg-Henriksson H, Zierath JR: 5-Aminoimidazole-4-carboxamide ribonucleoside treatment improves glucose homeostasis in insulin resistant diabetic (ob/ob) mice. *Diabetologia* 45:56-65, 2002

28. Halseth AE, Ensor NJ, White TA, Ross SA, Gulve EA: Acute and chronic treatment of ob/ob and db/db mice with AICAR decreases blood glucose concentrations. *Biochem Biophys Res Commun* 294:798-805, 2002

29. Buhl ES, Jessen N, Pold R, Ledet T, Flyvbjerg A, Pedersen SB, Pedersen O, Schmitz O, Lund S: Long-term AICAR administration reduces metabolic disturbances and lowers blood pressure in rats displaying features of the insulin resistance syndrome. *Diabetes* 51:2199-2206, 2002

30. Goodpaster BH, Kelley DE, Wing RR, Meier A, Taete FL: Effects of weight loss on regional fat distribution and insulin sensitivity in obesity. *Diabetes* 48:839-847, 1999

31. Salt IP, Johnson G, Ashcroft SJ, Hardie DG: AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release. *Biochem J* 335:533-539, 1998

32. Witters LA, Kemp BE: Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase. *J Biol Chem* 267: 2864-2867, 1992

33. Gamble J, Lopaschuk GD: Insulin inhibition of 5' adenosine monophosphate-activated protein kinase in the heart results in activation of

acetyl coenzyme A carboxylase and inhibition of fatty acid oxidation. *Metabolism* 46: 1270-1274, 1997

34. Beauloye C, Marsin AS, Bertrand L, Krause U, Hardie DG, Vanoverschelde JL, Hue L: Insulin antagonizes AMP-activated protein kinase activation by ischemia or anoxia in rat hearts, without affecting total adenine nucleotides. *FEBS Lett* 505: 348-352, 2001

35. Hickson-Bick DL, Buja ML, McMillin JB: Palmitate-mediated alterations in the fatty acid metabolism of rat neonatal cardiac myocytes. *J Mol Cell Cardiol* 32:511-519, 2000

36. Russell JC, Shillabeer F, Bar-Tana J, Lau DC, Richardson M, Wenzel LM, Graham SE, Dolphin PJ: Development of insulin resistance in the JCR:LA-cp rat: role of triacylglycerols and effects of MEDICA 16. *Diabetes* 47:770-778, 1998

37. Russell JC, Epling WF, Pierce D, Amy RM, Boer DP: Induction of voluntary prolonged running by rats. *J Appl Physiol* 63:2549-2553, 1987

38. Winder WW: Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. *J Appl Physiol* 91:1017-1028, 2001

39. Dengel DR, Pratley RE, Hagberg JM, Rogus EM, Goldberg AP: Distinct effects of aerobic exercise training and weight loss on glucose homeostasis in obese sedentary men. *J Appl Physiol* 81:318-325, 1996.

40. Russell JC, Amy RM, Manickavel V, Dolphin PJ, Epling WF, Pierce D, Boer D: Prevention of myocardial disease in the JCR:LA-corpulent rat by running. *J Appl Physiol* 66:1649-1655, 1989

41. Zhou YT, Grayburn P, Karim A, Shimabukuro M, Higa M, Baetens D, Orci L, Unger RH: Lipotoxic heart disease in obese rats: implications for human obesity. *Proc Natl Acad Sci USA* 97:1784-1789, 2000

42. Chiu HC, Kovacs A, Ford DA, Hsu F-F, Garcia R, Herrero P, Saffitz JE, Schaffer JE: A novel mouse model of lipotoxic cardiomyopathy. *J Clin Invest* 107:813-822, 2001

43. Corr PB, Creer MH, Yamada KA, Saffitz JE, Sobel BE: Prophylaxis of early ventricular fibrillation by inhibition of acylcarnitine accumulation. *J Clin Invest* 83:927-936, 1989

44. Regan TJ, Weisse AB: Diabetic cardiomyopathy. *J Am Coll Cardiol* 19:1165-1166, 1992

45. Zoneraich S, Mollura JL. Diabetes and the heart: state of the art in the 1990's. *Can J Cardiol* 9:293-299, 1993

CHAPTER 7. DISCUSSION AND CONCLUSIONS

CHAPTER 7. Discussion and Conclusions

The rapid increase in the incidence of obesity is contributing to the concurrent increase in insulin resistance and type 2 diabetes. Although there is a strong link between insulin resistance and cardiovascular disease, it is not clear whether insulin resistance develops in the heart or whether alterations in fatty acid metabolism contribute to insulin resistance. Furthermore, the rapid increase in obesity and insulin resistance is predicted to cause a concurrent increase in cardiovascular disease. Thus, the determination of whether the heart becomes insulin resistant and an understanding of the metabolic alterations occurring in cardiac insulin resistance would allow the development of clinical agents that target the heart and potentially prevent the development of cardiovascular disease.

Leptin Regulation of Cardiac Fatty Acid Metabolism

Leptin regulates fatty acid metabolism in liver, skeletal muscle and pancreas by partitioning fatty acids into oxidation rather than TG storage. The presence of leptin receptors in the heart suggests that leptin may also play a role in the regulation of cardiac fatty acid metabolism. We demonstrate for the first time that leptin activates fatty acid oxidation and decreases TG content in the heart (Chapter 3). However, the effects of leptin in the heart are independent of changes in the AMPK-ACC-malonyl-CoA axis. Thus, the mechanism by which leptin increases cardiac fatty acid oxidation remains unclear. Our studies show that leptin increases fatty acid oxidation, increases oxygen consumption and decreases cardiac efficiency. These effects of leptin may be explained by an increase in mitochondrial uncoupling protein activity. Indeed, the induction of mitochondrial uncoupling protein activity has been demonstrated to increase oxygen consumption (1) and is associated with an increase in fatty acid oxidation (2). However, an acute effect of leptin on mitochondrial uncoupling proteins has not yet been demonstrated.

The recent classification of obesity as an independent risk factor for cardiovascular disease and characteristic elevated leptin levels in obesity has lead to the suggestion that leptin may be the link between obesity and cardiovascular disease (3). Our data demonstrate that acute leptin treatment increases myocardial fatty acid oxidation rates and reduces cardiac efficiency. Chronic elevations in leptin also result in elevated fatty acid oxidation and TG depletion in skeletal muscle (4). Elevated rates of fatty acid oxidation have previously been demonstrated to reduce cardiac efficiency and the recovery of mechanical function following acute ischemia (5). Presently, it is not known whether leptin influences cardiac efficiency or the recovery of mechanical function following acute ischemia. However, given the ability of leptin to stimulate fatty acid oxidation acutely, it is possible that long-term elevations in plasma leptin levels may result in cardiac dysfunction by stimulating fatty acid oxidation and decreasing cardiac efficiency. This possibility remains to be tested.

Potential Mechanisms of Cardiac Insulin Resistance

The genetic deficiency of leptin receptors in the JCR:LA-cp rat results in TG accumulation in the heart, skeletal muscle and liver (Chapters 4 and 5). The accumulation of TG in the heart and skeletal muscle of the JCR:LA-cp rat is strongly associated with the development of insulin resistance. In the JCR:LA-cp rat heart, glycolytic rates are reduced by 50% and are directly proportional and associated with a 50% elevation in cardiac TG content (Chapter 4). Treatment with the lipid-lowering agent, MEDICA 16 prevents the accumulation of muscle TG along with the development of insulin resistance (5). Similarly, exercise-training improves whole body insulin sensitivity and the exercise-induced activation of AMPK is associated with a reduction in cardiac TG content and a stimulation of cardiac glycolysis (Chapter 6). The cessation of exercise-training and food restriction reverses the beneficial effects of exercise-training on cardiac metabolism. Thus, we have demonstrated that the JCR:LA-cp rat heart has a significant accumulation of TG that is associated with reductions in cardiac glucose utilization. Furthermore, in both skeletal muscle and heart, the accumulation of TG is associated with reduced glucose utilization and may be the mechanism responsible for the development of insulin resistance in the JCR:LA-cp rat.

The accumulation of TG can be the result of increased fatty acid supply, decreased fatty acid oxidation, or a combination of both. An increase in TG synthesis or decrease in TG lipolysis may also contribute to TG accumulation. We demonstrate that fatty acid oxidation is unaltered in isolated working JCR:LA-cp rat hearts under both fasting and fed nutritional states, suggesting that increased fatty acid supply rather than decreased fatty acid oxidation contributes to the accumulation of TG in this model of insulin resistance (Chapter 4). Indeed, both elevated plasma lipid levels and increased cytosolic H-FABP protein expression would indicate elevated fatty acid supply and increased fatty acid uptake. Of significance, treatment of JCR:LA-cp rats with lipid-lowering agents such as MEDICA 16 has previously been demonstrated to reduce plasma lipid levels, muscle TG accumulation and insulin resistance (6), suggesting that elevated fatty acid supply is a likely contributor to TG accumulation.

Consistent with the lack of changes observed in cardiac fatty acid oxidation, we observed no significant differences in the cardiac activities of AMPK, ACC, and CPT-I; key enzymes involved in the regulation of fatty acid oxidation (Chapter 4). We also demonstrate that skeletal muscle from JCR:LA-cp rats displays no significant difference in AMPK or ACC activities and lipid-lowering therapy with MEDICA 16 has no effect on the activity of these enzymes (Chapter 5). However, increases in hepatic ACC activity may contribute to the elevated plasma lipid levels because MEDICA 16 treatment resulted in a significant reduction in hepatic ACC activity. These data provide further support to the notion that elevated fatty acid supply contributes to TG accumulation in skeletal muscle and heart of JCR:LA-cp rats.

Although TG accumulation has been associated with contractile dysfunction (7-12), we did not observe changes in cardiac function in the JCR:LA-cp rat heart. We have postulated that the development of muscle insulin resistance requires a certain threshold level of TG accumulation. This may also be the case for the development of contractile dysfunction. Alternatively, elevations in fatty acid oxidation accompanying the accumulation of TG in obese Zucker rat hearts (7) could also explain the development of contractile dysfunction. Indeed, increased or decreased rates of cardiac fatty acid oxidation have been associated with contractile dysfunction (7,13,14). The relative contribution of TG accumulation and/or altered fatty acid oxidation to contractile dysfunction in insulin resistance is not currently known but remains an important question.

The role of TG accumulation in the development of cardiovascular disease is also not clear. We demonstrate that the development of insulin resistance in the JCR:LA-cp rat is accompanied by an elevation in cardiac TG content and a reduction in cardiac glycolysis (Chapter 4). Furthermore, exercise results in a reduction in cardiac TG content and a stimulation of cardiac glycolysis in the JCR:LA-cp rat (Chapter 6). Previous studies have demonstrated that exercise-training can prevent the development of cardiovascular disease in the JCR:LA-cp rat (15) suggesting that TG accumulation may contribute to cardiovascular disease in this model. Further studies are required to investigate the potential role of TG accumulation in the development of cardiovascular disease and contractile dysfunction.

Role of AMPK in Insulin Resistance and Potential Target for Treatment

AMPK plays a key role in the regulation of fatty acid and glucose metabolism. Not only would the hyperinsulinemia and hyperlipidemia characteristic of the insulin resistant syndrome be expected to reduce AMPK activity, reductions in AMPK could account for reduced glucose metabolism, decreased fatty acid oxidation and elevated TG content. Thus, we hypothesized that reductions in AMPK activity are associated with the altered fatty acid and glucose metabolism in the insulin resistant JCR:LA-cp rat heart. However, we did not observe a reduction in AMPK activity in the heart or skeletal muscle of insulin resistant JCR:LA-cp rats suggesting that alterations in muscle AMPK activity are not responsible for alterations in fatty acid and glucose metabolism during the development of insulin resistance. We have observed that AMPK activity is downregulated in type 2 diabetic (db/db) rat hearts suggesting that reductions in AMPK activity may be involved in type 2 diabetes. Furthermore, we observed that hepatic AMPK is significantly increased in the JCR:LA-cp rat and that MEDICA 16 treatment reduced the elevated hepatic AMPK activity (Chapter 5). The role of elevated hepatic AMPK in insulin resistance is not clear but may be a compensatory mechanism to inhibit increases in hepatic glucose production. This would provide an explanation for the lack of hyperglycemia in the JCR:LA-cp rat but requires further investigation.

There is enormous interest in the AMPK signaling cascade as a novel therapeutic target for the treatment of obesity, insulin resistance and type 2 diabetes (16-18). Indeed, exercise-training significantly improved whole body insulin sensitivity and the exercise-induced activation of cardiac AMPK was associated with a reduction in cardiac TG and a stimulation of cardiac glycolysis (Chapter 6). Furthermore, we demonstrate that exercise-training started at an early age prevents the development of whole body insulin resistance but weight gain following exercise-training reverses the improvement in insulin sensitivity. Treatment with the lipid-lowering agent, MEDICA 16, during the development of insulin resistance has also been shown to prevent the accumulation of muscle TG along with the development of insulin resistance (6). These data suggest that although alterations in AMPK do not appear to be responsible for the development of insulin resistance, the activation of AMPK may be capable of restoring the altered glucose and fatty acid metabolism characteristic of the insulin resistant JCR:LA-cp rat.

In summary, our studies demonstrate that leptin activates fatty acid oxidation and decreases TG content in the heart independent of changes in the AMPK-ACC-malonyl-CoA axis. Leptin receptor deficiency in the JCR:LA-cp rat heart results in TG accumulation resulting from elevated fatty acid supply rather than reduced fatty acid oxidation. The accumulation of cardiac TG is associated with reductions in cardiac glycolysis in the JCR:LA-cp rat heart. While chronic exercise improves whole body insulin sensitivity and the exercise-induced activation of cardiac AMPK was associated with a reduction in cardiac TG content and a stimulation of cardiac glycolysis, the beneficial effects of exercise are reversed following the cessation of exercise-training and food restriction.

Future Directions

The heart contains several isoforms of the leptin receptor (19). Studies are required to investigate which leptin receptor isoform mediates the increase in fatty acid oxidation and decrease in TG content. The effect of leptin on fatty acid metabolism in the db/db mouse heart, deficient in the long form of the leptin receptor, could provide information regarding the role of long form of the leptin receptor in the regulation of cardiac fatty acid metabolism.

Evidence suggests that leptin may increase fatty acid oxidation through induction of mitochondrial uncoupling activity (2). Studies are required to determine whether the induction of mitochondrial uncoupling activity is responsible for the elevation of fatty acid oxidation in the heart. If this is not the case, the signaling pathway by which leptin alters fatty acid oxidation will require further study.

The leptin-induced depletion of TG has been demonstrated to occur via increased lipolysis rather than increased esterification (4). One potential target of leptin is hormone sensitive lipase (HSL); however, further studies are necessary to determine whether leptin has acute effects on HSL activity.

Elevated rates of fatty acid oxidation have previously been demonstrated to reduce cardiac efficiency following acute ischemia (5). Whether leptin induction of fatty acid oxidation causes a further reduction in cardiac efficiency following acute ischemia requires further investigation. The elevated rates of fatty acid oxidation following acute ischemia have been attributed to an activation of AMPK (20). Whether the leptin induction of fatty acid oxidation is additive to AMPK activation of fatty acid oxidation would provide further insight into the regulation of cardiac fatty acid metabolism by leptin.

We concluded that the JCR:LA-cp heart has increased fatty acid uptake by demonstrating increased fatty acid supply and increased cytosolic H-FABP protein expression, a surrogate marker of fatty acid uptake. While direct measurements of fatty acid uptake into the JCR:LA-cp heart are required to verify this observation, this idea is consistent with studies demonstrating increased cardiac fatty acid uptake accompanied by increased expression of fatty acid transporters (CD36/FAT, pmFABP) in the plasma membrane (21). Investigations of the potential changes in fatty acid transporters (CD36/FAT, pmFABP) are still required in the insulin resistant JCR:LA-cp heart.

We demonstrate that the 12-week insulin resistant JCR:LA-cp heart has no significant change in cardiac work but a 50% reduction in cardiac glycolytic rates. Since the lack of difference in cardiac work would suggest that the heart maintains normal ATP production, further studies are required to investigate whether glycogen metabolism is compensating for the ATP production or whether alternate fuel sources such as lactate are being used by the insulin resistant heart.

Treatment with the lipid-lowering agent, MEDICA 16 has been shown to reduce plasma lipid levels, reduce muscle TG accumulation and prevent insulin resistance (6). Further studies are necessary to determine whether the lowering of plasma lipid levels would also reduce the accumulation of cardiac TG in the insulin resistant JCR:LA-cp rat.

The expression of a dominant-negative inhibitory mutant of AMPK in transgenic mice has demonstrated that the inhibition of AMPK completely blocks hypoxia- or AICAR-induced glucose uptake in muscle (22). However, the inhibition of AMPK only partially inhibited contraction-induced glucose uptake suggesting that other pathways may be involved in the exercise-induced benefits on insulin sensitivity (22). At present, specific AMPK activators are unavailable. Therefore, AICAR and exercise or contraction is widely used to investigate the role of AMPK activation on glucose and fatty acid metabolism. However, the specific activation of AMPK through AMPK activating agents or AMPK overexpression are required to verify the precise role of AMPK activation in the regulation of fatty acid and glucose metabolism.

Limitations

The measurement of intramuscular TG is often artifactually elevated because of the presence of extramuscular adipose tissue (23). To address this issue, visible adipose tissue was quickly dissected from the gastrocnemius muscle before freezing. Furthermore, the presence of intracellular TG in the gastrocnemius muscle of JCR:LA-cp rats has been previously demonstrated using electron microscopy and oil red O stain (6). New technology has also allowed the validation of intramuscular TG stores in vivo, using ^{1}H spectroscopy (24). Thus, although we cannot completely rule out the possibility that extracellular adipose tissue contributed to our measurements, our results are in agreement with several studies demonstrating a link between TG accumulation and the development of insulin resistance. Furthermore, the presence of adipocytes within the heart may also confound measurements of TG content. Visible adipose tissue was quickly removed from the heart prior to TG analysis. However, further studies are required to determine the exact contribution of cardiac adipocytes to measurements of TG content.

In the present studies, total ACC and AMPK were determined. While ACC-280 is the predominant isoform in the heart and muscle, ACC-265 is expressed, albeit at much lower levels. Since the enzymatic measurement of ACC activity cannot differentiate between the contributions of the different ACC isoforms, isoform specific changes in ACC activity would not be observed. Furthermore, while the $\alpha 2$ catalytic subunit of AMPK is predominant in heart and muscle, the $\alpha 1$ subunit is also expressed. The absence of specific antibodies to the catalytic subunits of AMPK limited our ability to determine isoform specific changes in AMPK activity.

We determined fatty acid oxidation rates in the JCR:LA-cp rat hearts using the isolated working heart preparation. Although our perfusion conditions were chosen to mimic the plasma levels of fatty acids, glucose and insulin in the JCR:LA-cp rat, other hormones such as leptin and adiponectin were not controlled for. Thus, while changes in fatty acid oxidation were not present in the isolated working JCR:LA-cp rat heart, it is not clear whether this is also the case *in vivo*.

We have demonstrated that the insulin resistant JCR:LA-cp rat hearts have a reduction in glycolytic rates in response to insulin. While glycolysis is one pathway of glucose metabolism, we have not considered the conversion of glucose to glycogen or TG or the potential shunting of glucose through the pentose-phosphate pathway. However, studies have demonstrated that a 32% suppression of glycolysis is associated with a significant reduction in whole body insulin stimulated glucose uptake (25). Still further experiments are required to directly measure glucose uptake as well as determine the amount of glucose going into glycogen, TG or the pentose-phosphate pathway.

In all cases, we determined enzyme activities under V_{max} conditions. Thus, any changes in enzyme activity related to changes in K_m would not have been detected.

References

---

1. Zhang CY, Hagen T, Mootha VK, Slieker LJ, Lowell BB: Assessment of uncoupling activity of uncoupling protein 3 using a yeast heterologous expression system. *FEBS Lett* 449:129-134, 1999

2. Lanni A, Mancini F, Sabatino L, Silvestri E, Franço R, De Rosa G, Goglia F, Colantuoni V: De novo expression of uncoupling protein 3 is associated to enhanced mitochondrial thioesterase-1 expression and fatty acid metabolism in liver of fenofibrate-treated rats. *FEBS Lett* 525:7-12, 2002

3. Leyva F, Godsland IF, Ghatei M, Proudler AJ, Aldis S, Walton C, Bloom S, Stevenson JC: Hyperleptinemia as a component of a metabolic syndrome of cardiovascular risk. *Arterioscler Thromb Vasc Biol* 18:928-933, 1998

4. Steinberg GR, Bonen A, Dyck DJ: Fatty acid oxidation and triacylglycerol hydrolysis are enhanced after chronic leptin treatment in rats. *Am J Physiol* 282:E593-E600, 2002
5. Liu Q, Docherty JC, Rendell JC, Clanachan AS, Lopaschuk GD: High levels of fatty acids delay the recovery of intracellular pH and cardiac efficiency in post-ischemic hearts by inhibiting glucose oxidation. *J Am Coll Cardiol* 39:718-725, 2002

6. Russell JC, Shillabeer G, Bar-Tana J, Lau DCW, Richardson M, Wenzel LM, Graham SE, Dolphin PJ: Development of insulin resistance in the JCR:LA-cp rat: Role of triacylglycerols and effects of MEDICA 16. *Diabetes* 47:770-778, 1998

7. Young ME, Guthrie PH, Razeghi P, Leighton B, Abbasi S, Patil S, Youker KA, Taegtmeyer H: Impaired long-chain fatty acid oxidation and contractile dysfunction in the obese Zucker rat heart. *Diabetes* 51:2587-2595, 2002

8. Zhou YT, Grayburn P, Karim A, Shimabukuro M, Higa M, Baetens D, Orci L, Unger RH: Lipotoxic heart disease in obese rats: implications for human obesity. *Proc Natl Acad Sci USA* 97:1784-1789, 2000

9. Chiu HC, Kovacs A, Ford DA, Hsu F-F, Garcia R, Herrero P, Saffitz JE, Schaffer JE: A novel mouse model of lipotoxic cardiomyopathy. *J Clin Invest* 107:813-822, 2001

10. Corr PB, Creer MH, Yamada KA, Saffitz JE, Sobel BE: Prophylaxis of early ventricular fibrillation by inhibition of acylcarnitine accumulation. *J Clin Invest* 83:927-936, 1989

11. Regan TJ, Weisse AB: Diabetic cardiomyopathy. *J Am Coll Cardiol* 19:1165-1166, 1992

12. Zoneraich S, Mollura JL. Diabetes and the heart: state of the art in the 1990's. *Can J Cardiol* 9:293-299, 1993

13. Belke DD, Larsen TS, Gibbs EM, Severson DL: Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (db/db) mice. *Am J Physiol* 279:E1104-E1113, 2000

14. Semeniuk LM, Kryski AJ, Severson DL: Echocardiographic assessment of cardiac function in diabetic db/db and transgenic db/db-hGLUT4 mice. *Am J Physiol* 283:H976-H982, 2002

15. Russell JC, Amy RM, Manickavel V, Dolphin PJ, Epling WF, Pierce D, Boer D: Prevention of myocardial disease in the JCR:LA-corpulent rat by running. *J Appl Physiol* 66:1649-1655, 1989
16. Moller DE: New drug targets for type 2 diabetes and the metabolic syndrome. *Nature* 414:821-827, 2001

17. Winder WW: AMP-activated protein kinase: possible target for treatment of type 2 diabetes. *Diabetes Technol Ther* 2:441-448, 2000

18. Winder WW, Hardie DG: AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* 277:E1-E10, 1999

19. Tartaglia LA: The leptin receptor. *J Biol Chem* 272:6093-6096, 1997

20. Kudo N, Gillespie JG, Kung L, Witters LA, Schulz R, Clanachan AS, Lopaschuk GD: Characterization of 5' AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. *Biochim Biophys Acta* 1301:67-75, 1996

21. Luiken JF, Arumugam Y, Dyck DJ, Bell RC, Pelsers MM, Turcotte LP, Tandon NN, Glatz JF, Bonen A: Increased rates of fatty acid uptake and plasmalemmal fatty acid transporters in obese Zucker rats. *J Biol Chem* 276:40567-20573, 2001

22. Mu J, Brozinick JT Jr, Valladares O, Bucan M, Birnbaum MJ: A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* 7:1085-1094, 2001

23. Guo Z, Mishra P, Macura S: Sampling the intramyocellular triglycerides from skeletal muscle. *J Lipid Res* 42:1041-1048, 2001

24. Szczepaniak LS, Babcock EE, Schick F, Dobbins RL, Garg A, Burns DK, McGarry JD, Stein DT: Measurement of intracellular triglyceride stores by ^{1}H spectroscopy: validation *in vivo*. *Am J Physiol* 276:E977-E989, 1999

25. Kim JK, Youn JH: Prolonged suppression of glucose metabolism causes insulin resistance in rat skeletal muscle. *Am J Physiol* 272:E288-E296, 1997